

Evidence of kinetic proofreading through allergen specific IgE at the human mast cell IgE receptor

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Abstract

Background Activation of mast cells through IgE results in secretion and shedding of mast cell proteins and in vivo models suggest that these processes are governed by IgE antibody affinity. **Methods** We passively sensitized cultured primary human mast cells with recombinant human IgE clones with either high or low affinity for Der p 2, with a 200-fold affinity difference, and activated them with recombinant allergen. Activation was assessed by CD63 upregulation and PGD₂ secretion. Supernatants collected from mast cells activated for 0, 3, 6 and 24 hours were assessed for PGD₂ and inflammatory mediators on the OLINK platform at repeated time points. **Results** CD63 upregulation and PGD₂ synthesis scaled with affinity, as did secretion of cytokines like IL-8 and IL-13. Secretion of chemokines like CCL3 and CCL4 appeared to depend less on affinity, whereas shedding of surface markers CD40, SLAMF4 and CD5, and secretion of intracellular markers SIRT2 and CASP-8, were elevated by stimulation through low affinity IgE compared with high affinity IgE, illustrating differential responses dependent on the affinity of IgE. **Conclusion** Cytokine secretion and shedding of surface receptors of sensitized, cultured primary human mast cells is differentially regulated depending on the affinity of IgE for the Der p 2 allergen and may shape the chronic response to repeated allergic activation.

Evidence of kinetic proofreading through allergen specific IgE at the human mast cell IgE receptor

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Contribution:

HJH, PAW and CH designed the study, CH performed mast cell experiments, JJ performed data analyses, LA, SED, DF and CW performed measurements, LHC contributed essential reagents. CH, JJ and HJH drafted the manuscript. All authors revised the manuscript and will approve the final version.

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Abstract (208 words)

Background

Activation of mast cells through IgE results in secretion and shedding of mast cell proteins and in vivo models suggest that these processes are governed by IgE antibody affinity.

Methods

We passively sensitized cultured primary human mast cells with recombinant human IgE clones with either high or low affinity for Der p 2, with a 200-fold affinity difference, and activated them with recombinant allergen. Activation was assessed by CD63 upregulation and PGD₂ secretion. Supernatants collected from mast cells activated for 0, 3, 6 and 24 hours were assessed for PGD₂ and inflammatory mediators on the OLINK platform at repeated time points.

Results

CD63 upregulation and PGD₂ synthesis scaled with affinity, as did secretion of cytokines like IL-8 and IL-13. Secretion of chemokines like CCL3 and CCL4 appeared to depend less on affinity, whereas shedding of surface markers CD40, SLAMF4 and CD5, and secretion of intracellular markers SIRT2 and CASP-8, were elevated by stimulation through low affinity IgE compared with high affinity IgE, illustrating differential responses dependent on the affinity of IgE.

Conclusion

Cytokine secretion and shedding of surface receptors of sensitized, cultured primary human mast cells is differentially regulated depending on the affinity of IgE for the Der p 2 allergen and may shape the chronic response to repeated allergic activation.

Introduction (text 3113 words, 31 refs, 5 figures, 2 tables)

Signaling mediated by antibodies and their receptors through ITAM motifs has been shown to be subject to molecular editing through kinetic proofreading in mouse and rat models, resulting in distinct responses of myeloid effector cells depending on the affinity of the immunoglobulin-antigen interaction(1,2). Activation with low affinity antigen reduces leukotriene and cytokine (TNF- α , IL-6, IL-13) production but enhances production of chemokines CCL2, CCL3 and CCL4. This is reversed in cells activated with high affinity antigen (2). We hypothesize that human mast cells stimulated through high- and low affinity IgE respond similarly.

The generation of cytokines and chemokines from activated mast cells is a result of de novo synthesis and release of these soluble mediators. Ectodomain shedding of membrane proteins is another, more rapid, mechanism of control of membrane proteins that can lead to generation of soluble messengers and feedback loops (3). This process is, however, uncharted on mast cells during activation. CD5, CD6, CD40 (4) and SLAMF4 (5) are regulatory molecules expressed on mast cells. CD5 has not been identified in mast cells by immunohistochemistry (6), but activity at the CD5 promoters has been documented recently (7). Differential regulation of these surface markers may affect mast cell function significantly.

We have previously shown that, in the immediate allergic response, reactivity increases threefold and sensitivity (EC₅₀) increases 15000-fold in cultured human mast cells (MC) sensitized with two low affinity IgE clones compared with two high affinity IgE clones specific for the house dust mite *Dermatophagoides pteronyssinus* class two allergen (Der p 2) (8,9). A tenfold increase in the affinity of IgE clones contributing to complex formation resulted in 19% increase in mast cell reactivity (9).

Here we present evidence using the major allergen Der p 2 and cloned pairs of IgE molecules specific for Der p 2 with affinity differing 200 fold (8,9), that a broad range of cytokine and chemokine expression as well as shedding of surface receptors of primary cultured human mast cells, depends on the affinity of IgE for allergen. This is the first documentation of molecular editing due to variation in affinity of the cellular response in human mast cells and suggests that measures of quantity of allergen specific IgE (sensitization) is inadequate without knowledge of the affinity of the interaction between IgE and its cognate allergen. In previous work, the significance of complexity and composition of IgE clones has been illustrated (8).

Methods

Mast cell culture

CD133⁺ stem cells purified from buffy coats were cultured for 8 weeks to become mature mast cells as described(10)·(11)·(12). The regional ethics committee approved use of buffy coats for mast cell research (#63, 1995).

IgE antibodies for mast cell sensitization

After 6 weeks of culture each cell line was divided in two aliquots that were sensitized with 80 kU/L recombinant human IgE (rIgE) containing 7% each of non-overlapping Der p 2-specific IgE clones in combination with 86% of a non-allergen-specific IgE antibody (8); clones H10:H12 (kD 284 nM) and P4C (kD 291 nM) for low affinity stimulation and clones H12 (kD 1.12 nM) and P4E (kD 1.4 nM) for high affinity stimulation. The difference in affinity of IgE between the conditions was thus approximately 200-fold.

Mast cell activation and generation of supernatant samples

After 8 weeks of culture, IgE sensitized mast cells were activated by challenge with recombinant Der p 2 allergen in log₁₀dilutions for 30 minutes in a water bath at 37° C. Activation was assessed by flow cytometry as surface expression of CD63. Supernatants were generated by activating MCs for 3, 6 or 24 hours at 37° C with an optimal concentration of Der p 2 allergen determined above. After centrifugation for 10 minutes, 4° C, 2000G, the supernatant and pellet were frozen separately. A supernatant sample not activated with allergen (time 0 hours) was saved as baseline control.

Assessment of PGD₂ release

Release of PGD₂ was monitored by the use of the PGD₂-MOX EIA kit from Cayman Chemicals (Ann Arbor, Michigan, USA) as instructed by the manufacturer. In addition, release of PGD₂ and its metabolites was validated in a separate experiment by quantitative UPLC/MS-MS as described (13).

Measurement of cytokines and chemokines in the supernatant

Soluble mediators were assessed using the Inflammation panel from the OLINK Proteomics platform(14) in the supernatant (n = 4) at 0 hours (resting state) and at 3, 6 and 24 hours after activation with high and low-affinity IgE. Samples only containing cell culture medium were analyzed as a blank control. The normalized protein expression values used are calculated from Ct values of a proximity extension assay that has been shown to have superior sensitivity compared to other detection methods (15). Sensitivity and range of detection for each analyte is available on OLINKs home page.

Statistics

The fraction of mast cells expressing CD63 was plotted against the logarithm of allergen concentrations. The maximal reactivity (%CD63⁺ mast cells) and mast cell sensitivity (EC₅₀) were estimated by four-

parameter logistic regression. The results for mast cell reactivity and sensitivity are given as median and interquartile range (IQR). The statistical significance of reactivity, sensitivity, prostaglandin D2 synthesis and soluble mediator expression was analyzed using 2way ANOVA. GraphPad Prism v5 was used for statistical calculations.

Normalized Protein Expression (NPX, Olink’s arbitrary unit in log2) values from the biomarkers that passed quality control (NPX value > +/- 0.3 compared to medium control), were used as input for differential expression analysis using the R Bioconductor package *limma*. Using this package, a model design was implemented to test for differential expression between non-activated mast cells (0 h) and activated mast cells (3 h, 6 h and 24 h respectively), while correcting for donor variability (cell line number) (16). For the analysis of the PEA assay, *limma* uses an empirical Bayesian method that considers the variance across all biomarkers to estimate per biomarker variance for model estimation. Contrasts were used to conduct comparisons of interest and generate p-values from the moderated t-test. $P < 0.05$ was considered significant.

Results

Reactivity and sensitivity of mast cell degranulation and Prostaglandin D₂ synthesis increase with increasing affinity of IgE for allergen.

Mast cell reactivity was threefold higher with high affinity IgE clones (65%; IQR = 47 – 80) than with low affinity clones (20%; IQR = 12 – 25, $p=0.0081$) (figure 1A). Mast cell sensitivity (EC_{50}) decreased 12.850-fold from 0.07 pg/ml (IQR = 0.02 – 7.2) Der p 2 allergen with high affinity clones to 900 pg/ml (IQR = 0.3 – 1.1) Der p 2 allergen when low affinity clones were used for sensitization ($p=0.0395$). Release of prostaglandin D2 assessed at 0, 3, 6 and 24 hours by EIA developed with significance for time ($p<0.007$) and affinity ($p=0.0437$) compared with baseline ($n=3$, Figure 1B). The release of PGD_2 and its metabolites PGJ_2 , δ -12- PGJ_2 and 15-deoxy- δ -12,14- PGJ_2 was validated by mass spectrometry in a separate cell line (Figure 1, c-f). Four-fold more PGD_2 was synthesized with high affinity IgE than with low affinity IgE activation after 3 ($p=0.0024$) and 6 ($p=0.0188$) hours ($n=4$).

Soluble mediator release by high and low IgE affinity activation

Amongst the 54 mediators detected (table 1), 19 did not change expression in response to IgE activation, among them IL-4, IL-6 and SCF, that were added to the culture medium. 37 mediators were not detected. Among the remaining substances that responded to change in affinity of IgE 24 could be grouped into 3 types of proteins: 10 soluble cytokines and chemokines and 3 soluble cytoplasmic proteins (Figure 2, Table 2), 9 Type I Transmembrane molecules including cytokine receptors and membrane-bound cytokines and 2 Type II Transmembrane molecules (Figure 3, Table 2). A further 6 cytokines and chemokines, 3 transmembrane proteins and 3 cytoplasmic proteins were detected but did not vary significantly (Figure 4). Mediators are presented in order of strength of response in figure 2 and figure 3. In figure 5, momentary distributions of reactants measured at 3, 6 or 24 hours are plotted.

Cytokine and chemokine response

CCL2, CCL3, IL-8, OSM and LIF were expressed at high levels at baseline (Table 1). IL-13, IL-8, LIF, HGF, and CCL2 (Figure 2, a-c, e, g) and membrane bound CSF-1 and FL3L (Figure 3, h, i) were only upregulated through high affinity IgE activation at all times investigated. OSM, CCL3 and CCL4 were released by stimulation through both low- and high affinity IgE activation at 3 hours, but at higher levels for high affinity IgE (Figure 2, d, h, i). Production through high affinity IgE activation of CCL3 and CCL4 had ceased at 24 hours. CCL7/MCP3 (Figure 2, j) was significantly upregulated through low affinity IgE activation.

Cytoplasmic markers in the supernatant

CASP-8 (Figure 2, k) was significantly upregulated with low affinity IgE and differentially regulated with

low- and high affinity IgE activation. SIRT2 (Figure 2, l) was significantly upregulated with low affinity IgE but not with high affinity IgE activation. STAMPB (Figure 2, m) was upregulated through high affinity IgE activation only.

Shedded type I and type II transmembrane molecules

CD40 and CD5 (Figure 3, a, b) were elevated through low affinity IgE after 6 and 24 hours. CD40, CD5, SLAMF4, IL10RB, CD6 and TGF-a were differentially regulated with low- and high affinity IgE. PD-L1 was upregulated by both low- and high affinity IgE (Figure 3, g).

The soluble TMII molecules TNFSF12 and TNFSF14 (Figure 3, j, k) were upregulated primarily through activation with high affinity IgE at all time points, while low level upregulation by low affinity IgE activation happened later.

Discussion

We hereby confirm that increase of IgE affinity increases both reactivity and sensitivity of degranulation measured as CD63 activation, and de novo synthesis of lipid mediators measured as the release of PGD₂ and its metabolites by human mast cells (Figure 1). Furthermore, we confirm results obtained in murine models, that IgE affinity directs the soluble mediator expression from activated mast cells(2) which may dictate the late phase response in vivo. Activation of human mast cells through high affinity IgE induced secretion of cytokines and chemokines, whereas stimulation through low affinity IgE resulted primarily in the release of membrane bound receptors.

The immediate degranulation response

Basophil reactivity has been associated with severity of allergic symptoms while sensitivity has been correlated with the allergen threshold(17)·(18). Increasing affinity increases both reactivity and sensitivity of a mast cell response (Figure 1A). Previous work has shown that clonality of IgE and the relative concentrations of IgE clones play a role in basophil activation (8) as well as in mast cell activation (9). We find that PGD₂ is secreted by mast cells with increased sensitivity and reactivity when activated by high affinity IgE with a peak at 3 hours (Figure 1b), and that metabolites PGJ₂, delta-12-PGJ₂ and 15-deoxy-delta-12, 14 PGJ₂, accumulate at later time points to support this finding (Figure 1 c-f).

Late phase response of protein secretion; difference between the low and high affinity activation

In order to assess mediator release during mast cell activation by either low- or high affinity stimulation, we analyzed the expression of 91 soluble inflammation markers in the supernatant of mature mast cells after 3, 6 and 24 hours of activation and detected 54 of these at at least one of the four time points sampled. The early response after 3 hours was dominated by cytokines IL-8, IL-13, LIF, HGF and CCL4 produced due to high affinity IgE crosslinking. PD-L1 was induced by both high and low affinity and with no significant difference. A number of shedded surface markers (TNFSF12, TNFSF14, CSF-1, TGF-a) and the cytosolic protein STAMPB, were induced earlier and more persistently by high affinity IgE than by low affinity IgE.

Most effects of signalling through low affinity IgE appeared after 6 hours, and many markers detected after low affinity activation were shared with high affinity signalling. The cytosolic proteins CASP8 and SIRT2 were specifically induced by low affinity IgE at 3 hours. The markers that most notably appeared to be specific for low affinity signalling were shedded CD40, SLAMF4 and CD5. This is consistent with the observations that low affinity IgE signalling has slower onset than high affinity signalling (2). CD40 is involved in the somewhat controversial antigen presentation of mast cells (4). In mast cells, CD5 may not be expressed in the same way as it is on T cells (6), but transcriptional activity at the promoter suggests it is expressed(7). SLAMF4 is expressed as a regulatory molecule on mast cells (5) that may have inhibitory as well as activating functions. Extensive shedding after 6 hours of these molecules as well as IL10-RB and CD6 and significantly more release of CCL3, TGF-b and CASP-8 differentiates the low affinity response of human mast cells from a high affinity response. One may speculate that the phenotype of the mast cell now adapts to the low

affinity stimulus. In addition, the membrane receptors released become soluble signals that may modulate the physiologic response.

CSF-1, Fit3L, TGF- α , CD40 and TNFSF11 are known to be targets of ADAM17 that is expressed in mast cells, but there was no clear association of ADAM17 and affinity of IgE or timing of release. CD6 and its ligand CD318 (CDCP1) were shedded after low affinity IgE stimulation at 6 and 24 hours, respectively. While IL-18 and IL-10 were released at 3 and 6 hours under conditions of high affinity IgE, IL-18R1 and IL-10Rb were shedded under low affinity IgE conditions at 6 and 24 hours, suggesting the presence of a feedback loop. Shedding of a receptor may be a method to quickly change the phenotype of the cell by releasing the surface receptor, or to generate a signal molecule, or both (3).

Effect of mast cell derived mediators

LIF, OSM, CCL2, CCL3 and CCL4 were released both by high and low affinity IgE signalling, with CCL3 being the only mediator still differentially upregulated by low affinity stimulation 24 hours after activation. This is in contrast to comparable results from a murine response at six hours, where CCL2, CCL3 and CCL4 were predominantly induced by low affinity IgE (2). Possible explanations for this are the lesser difference in IgE affinity in our model (200-fold) than in the murine model (1600-fold), and the inbred nature of the murine model compared with the outbred human mast cell cultures derived from independent individuals. Moreover, the extreme difference in affinity of the murine system is due to different antigens, and not due to difference in antibody affinity for the same allergen, which is used in our system employing well characterized recombinant human IgE clones.

The affinity related differential mediator response was demonstrated to have functional consequence in the previously mentioned mouse model; the distinct signaling profile including IL-8 elicited from the high affinity stimulation accumulated more neutrophils than monocytes/macrophages whereas the low affinity stimulation recruited fewer neutrophils and more monocytes(2). Increased amounts of CCL2, CCL3 and CCL4 have been measured in BAL of patients with severe asthma compared with controls(19,20). Our data suggests that these chemokines could be derived from activated mast cells as part of their modulation of the late phase response.

The presence of neutrophils in induced sputum or BAL of patients with allergic asthma is associated with severe, chronic asthma (21), but the relationship of neutrophils to mast cell induced T2 disease is less clear. The murine study and our human data suggest that sputum neutrophilia may also be explained by high affinity IgE in addition to corticosteroid use or Th1/Th17 immunity (22). Neutrophils release elastase which activates matrix metalloproteinases (MPPs) and degrades extracellular matrix proteins such as collagens to contribute to the development of chronic airway inflammation (23). IL-8 recruits and activates neutrophils during the late phase reaction(24) and elevated levels of IL-8 have been found in BAL fluid from asthmatic patients(25). We observed a significant increase in IL-8 with high affinity IgE compared to low affinity IgE and baseline. Considering the strong effect of IL-8 on neutrophils, our results indicate that the presence of high affinity IgE antibodies may increase the number of neutrophils and thereby the risk for developing chronic allergic inflammation. The role of monocytes/macrophages in allergic asthma is less well understood (26). IL-13 is secreted by Th2 cells, mast cells and basophils(27). It induces IgE class-switch recombination in B-cells and activates, recruits and promotes survival of mast cells and eosinophils(28). Consistent with results from BMMCs(2) we observed a significant increase in IL-13 with high affinity IgE stimulation compared to low affinity IgE and baseline. (29) These observations suggest that mast cells may have a role in both neutrophil, eosinophil and granulocytic asthma, and that affinity of IgE for allergen may explain the shift from one endotype to another.

Effects beyond the late phase

The detected amounts of IL-8, IL-13 and LIF are still significantly different from baseline 24 hours after allergen activation ($p < 0.001$) (figure 2, a-c). This could indicate an effect of these cytokines that goes beyond the late phase response (6-8 hours) and possibly adds to the development of chronic inflammation. Increased cytokine secretion may be associated with asthma severity as is supported by studies showing higher levels of IL-8 and IL-13 in patients with chronic severe asthmatic disease in addition to a negative

correlation of cytokine secretion with lung function (20,30,31) . Several cytokines novel to the mast cell were also significantly upregulated by high affinity IgE (CSF-1, FLT3L, HGF, OSM and IL-12b). CSF-1 and Flt3L may be involved in maintenance and activation of hematopoietic progenitors, and HGF supports angiogenesis and tissue regeneration of epithelial cells. OSM remains poorly defined, but closely related to IL-6 and LIF.

When combining the current results with data from animal models(1,2) mast cells seem to be able to sense differences in IgE affinity and this may have important implications for their role in shaping early and late phase allergic responses and may even impact chronic asthma by bridging between different endotypes dominated by immune cell subsets favored by different mast cell mediators. Clearly, additional experiments are needed to substantiate the direct effects of molecular editing through kinetic proofreading in human as well as mouse mast cells and its downstream effects. Moreover, links between IgE affinity and asthma endotypes may be followed up in allergic individuals with primarily high vs low affinity IgE towards well characterized allergens.

Conclusion

Activation through high affinity IgE leads to well-characterised cytokine release. When activating primary human mast cell lines through low affinity IgE with the native allergen Der p 2, some chemokines, and cytoplasmic proteins were released, and surface receptors were solubilized. The impact of finely tuned IgE affinity for allergen to the nature of the mast cell response in both cytokine and chemokine production as well as surface marker profile may modulate the severity of the late phase response and determine the chronic response to repeated allergic activation.

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Figure legends

Figure 1

a: Mast cell activation as CD63 expression after stimulation with recombinant Der p 3 allergen for 30 min at 37C at high (red) and low (blue) IgE affinity.

b: Prostaglandin D₂ synthesis from 3 independent cell lines at high (red) and low (blue) IgE affinity. (c-f) Small graphs chart the metabolism of PGD₂ through PGJ₂ and delta-12-PGJ₂ to 15-deoxy-delta-12,14-PGJ₂ detected by MS of one cell line.

Figure 2:

Plots of cytokines and chemokines and secretory proteins up-regulated significantly by high-affinity (red) compared to low-affinity (blue) activation, or, low-affinity compared to high-affinity activation after activation. The upregulation is shown as fold change compared with resting (0 hour) mast cells,. Ordinates are dimensioned to illustrate the particular reactants variation. An * next to the reactant indicates significance in the limma analysis, a ^H and ^L indicate that a marker is upregulated significantly with high or low affinity IgE. Detailed information is given in table 1.

Figure 3:

Plots of shedded surface markers up-regulated significantly by high-affinity (red) compared to low-affinity (blue) activation, or, low-affinity compared to high-affinity activation after activation. The upregulation is shown as fold change compared with resting (0 hour) mast cells,. Ordinates are dimensioned to illustrate the particular reactants variation. An * next to the reactant indicates significance in the limma analysis, a ^H and ^L indicate that a marker is upregulated significantly with high or low affinity IgE. Detailed information is given in table 1.

Figure 4

Secretory proteins detected at different timepoints after activation. The up-regulation is shown as the fold change compared to corresponding resting mast cells (0 hour).

Figure 5

An alternate visualization of the detection of reactants in mast cell supernatants after stimulation through either high or low affinity IgE. The up-regulation is shown as the fold change compared to corresponding resting mast cells (0 hour). At 24 hours the range of upregulation is only 8-fold, where it is 64-fold at 3 and 6 hours.

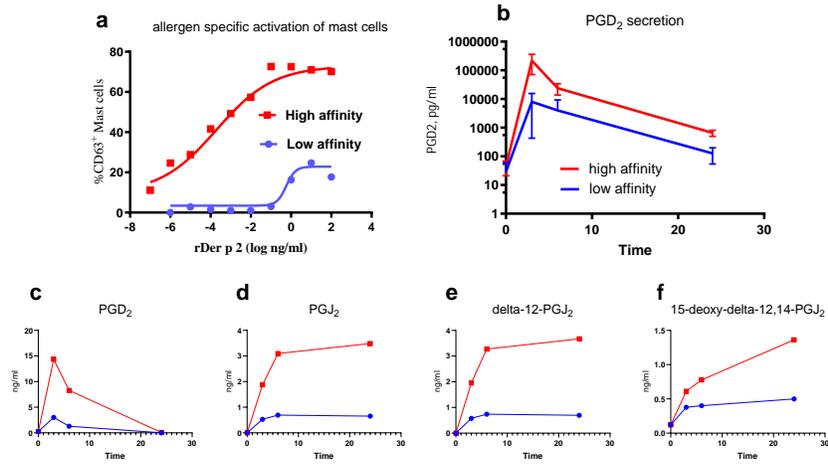
Table Legends

Table 1

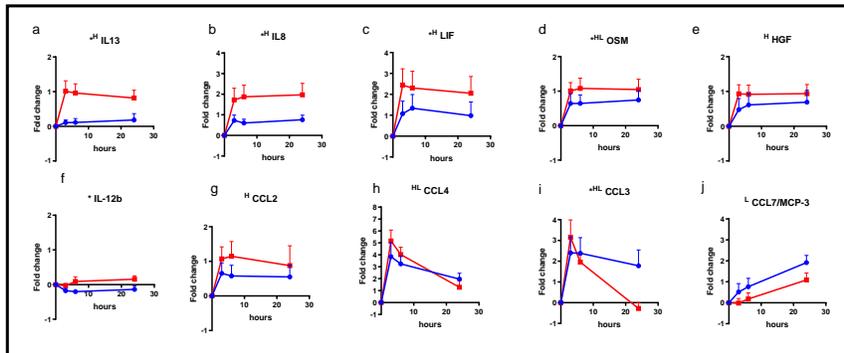
Mean Olink NPX data for all measured reagents at all time points.

Table 2

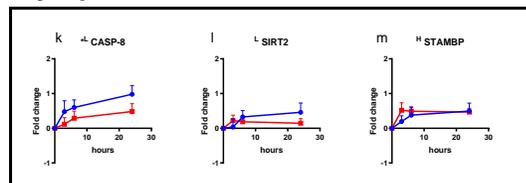
Results of a limma analysis of changes over time of mast cells activated through either high- or low affinity IgE.



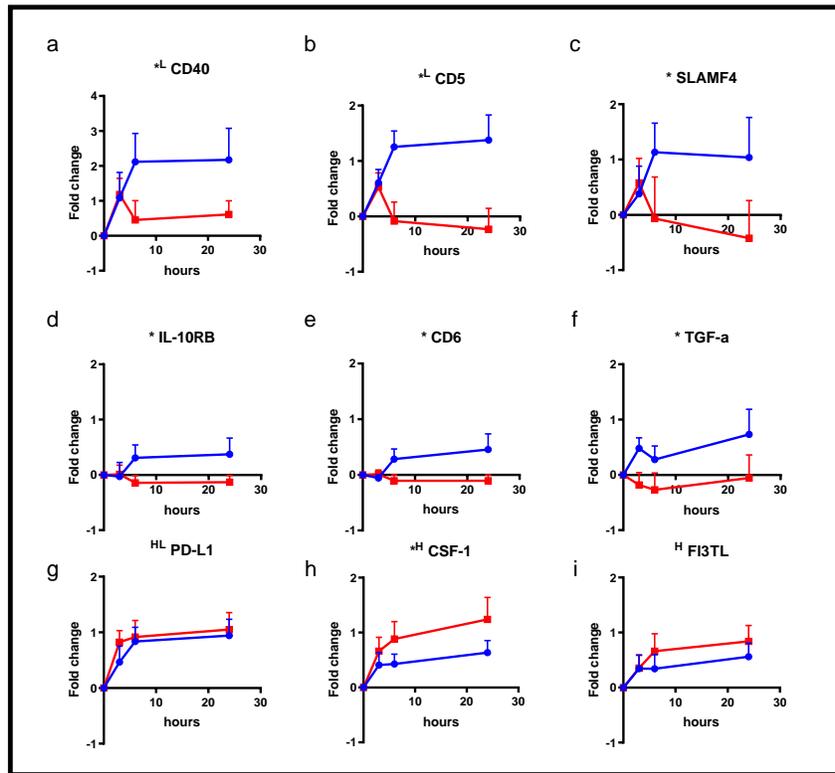
Cytokines & Chemokines



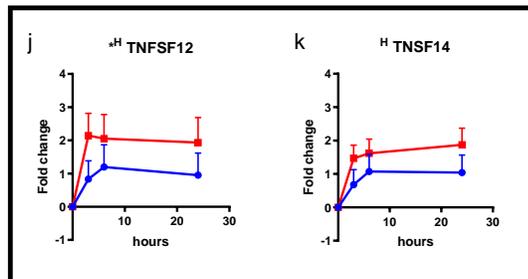
Cytoplasmic Proteins



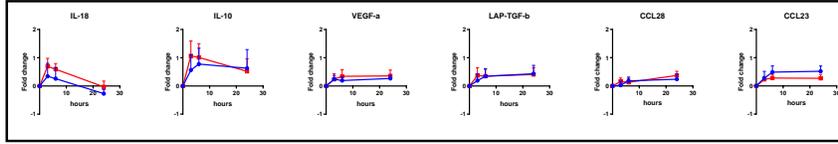
Type I Transmembrane Proteins



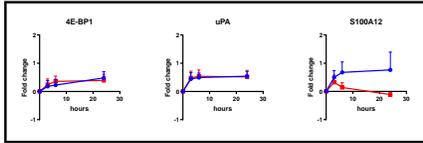
Type II Transmembrane Proteins



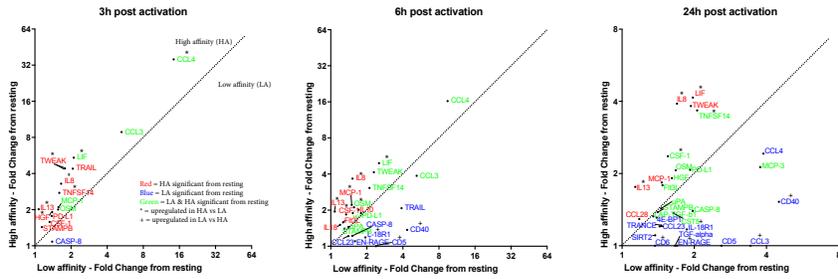
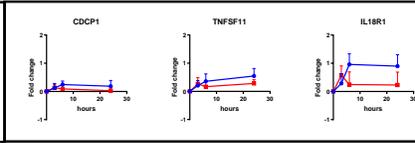
Cytokines & Chemokines



Cytoplasmic Proteins



Type I/II Transmembrane Proteins



| | Low affinity 0 hours | | | | High affinity 0 hours | | | |
|--------------|----------------------|------------|------------|------------|-----------------------|------------|------------|------------|
| | 0 hours | 3 hours | 6 hours | 24 hours | 0 hours | 3 hours | 6 hours | 24 hours |
| MCP-1 | 8.40488352 | 9.058985 | 8.3861874 | 8.0565653 | 8.2741675 | 9.342649 | 9.419378 | 9.1494489 |
| OSM | 8.12419677 | 8.76673 | 8.767239 | 8.8675685 | 8.10213242 | 9.1060728 | 9.181756 | 9.148376 |
| LIF | 7.91567458 | 8.988223 | 9.2526401 | 8.89227 | 7.743918316 | 10.183057 | 10.047153 | 9.7958411 |
| CD40 | 6.26196419 | 7.3446447 | 8.3796985 | 8.4344743 | 5.79605816 | 6.978249 | 6.9493121 | 6.405191 |
| MMP-1 | 5.81147911 | 6.6512713 | 6.8761314 | 6.8719764 | 6.87294863 | 7.4984154 | 7.227296 | 7.225484 |
| HGF | 5.800536183 | 6.2776168 | 6.412687 | 6.4909527 | 5.755406422 | 6.6881824 | 6.6744138 | 6.692784 |
| TWEAK | 5.315714024 | 6.1473941 | 6.5121635 | 6.2685009 | 4.99362086 | 7.1361395 | 7.0449103 | 6.9285401 |
| uPA | 4.265262154 | 4.7089973 | 4.7375678 | 4.8072799 | 4.27676359 | 4.7541456 | 4.8147705 | 4.7920764 |
| TRAIL | 3.825427442 | 4.8742619 | 5.7879972 | 5.1838204 | 3.46152527 | 5.596775 | 4.5162828 | 3.941466 |
| FN31 | 3.58161897 | 3.9258606 | 3.9243064 | 4.1419503 | 3.346296148 | 3.7007955 | 4.0065486 | 4.1847171 |
| CC13 | 3.571782406 | 5.9717401 | 5.95417 | 5.3505326 | 4.227960708 | 7.3769881 | 6.1800492 | 3.9444762 |
| IL10 | 3.54532054 | 4.1077733 | 4.1199404 | 4.171161 | 3.49317853 | 4.5048209 | 4.4491062 | 3.9771895 |
| IL8 | 3.123782533 | 3.8496546 | 3.7261747 | 3.8853919 | 3.447566966 | 5.1676034 | 5.3221929 | 5.4161612 |
| PD-1 | 2.685032429 | 3.1501935 | 3.5211921 | 3.6274288 | 2.590384489 | 3.4118974 | 3.5069235 | 3.6407296 |
| CSF-1 | 2.566480163 | 2.9737337 | 2.9038009 | 3.2012001 | 2.22399706 | 2.9848593 | 3.2064241 | 3.5638798 |
| TNFSF14 | 1.947441537 | 2.6247382 | 3.0199718 | 2.987357 | 1.75566684 | 3.2205973 | 3.3711143 | 3.6298896 |
| CD244 | 1.90961372 | 2.2883143 | 3.0425751 | 2.946441 | 1.693879074 | 2.2707121 | 1.6264339 | 1.27149 |
| MMP-10 | 1.717236187 | 2.1822296 | 2.02875 | 2.2031852 | 1.546077868 | 1.6041675 | 1.8975088 | 2.0126667 |
| IL-18R1 | 1.402832927 | 1.6908579 | 2.3633446 | 2.2993404 | 0.872697312 | 1.4451182 | 1.1173286 | 1.0996933 |
| VEGFA | 1.311519496 | 1.5503476 | 1.5025678 | 1.5768214 | 1.190963119 | 1.427901 | 1.5327316 | 1.545849 |
| CSY5 | 0.980480554 | 1.3462975 | 1.3421552 | 1.6939634 | 0.912092608 | 1.178902 | 1.2863882 | 1.3643625 |
| CC123 | 0.94038145 | 1.2070549 | 1.4296289 | 1.4032347 | 0.939800234 | 1.13701608 | 1.2173285 | 1.2069258 |
| MCP-3 | 0.803581521 | 1.147099 | 1.5694405 | 2.7178745 | 0.918022588 | 0.9035474 | 1.0984624 | 2.0076232 |
| ADA | 0.700415224 | 1.1172379 | 1.0232143 | 0.4650387 | 0.531068059 | 0.9665553 | 0.9356077 | 0.3298688 |
| TRANCE | 0.668042888 | 0.8749575 | 1.024137 | 1.2123262 | 0.7065089 | 0.9704944 | 0.8719616 | 0.9858537 |
| CC128 | 0.565737331 | 0.5911551 | 0.7442027 | 0.8037373 | 0.460842637 | 0.6194814 | 0.5888346 | 0.8306858 |
| IL18 | 0.554810911 | 0.8986897 | 0.8137399 | 0.281534 | 0.247476559 | 0.9443381 | 0.8387257 | 0.2144364 |
| IL-10RB | 0.544771196 | 0.515852 | 0.8526073 | 0.9154818 | 0.258589734 | 0.368475 | 0.1151867 | 0.1273381 |
| CC15 | 0.474648445 | 0.4005723 | 0.5710297 | 0.8223659 | 0.359203415 | 0.3792425 | 0.5097738 | 0.5335947 |
| CD5 | 0.436874466 | 1.0411768 | 1.6918945 | 1.8142787 | 0.603362695 | 1.1314477 | 0.5170063 | 0.3711225 |
| CC14 | 0.364480908 | 4.206073 | 3.6064552 | 3.2318609 | 0.549759039 | 5.7104435 | 4.5712764 | 3.8251116 |
| MCP-2 | 0.329782164 | 0.6513372 | 0.9246132 | 0.2084854 | 0.054881843 | 0.1139195 | 0.2507969 | 0.0920416 |
| SIRT2 | 0.309753617 | 0.3448238 | 0.6354182 | 0.7687622 | 0.162003081 | 0.3850273 | 0.3496818 | 0.3063572 |
| STAMBP | 0.291000933 | 0.4836145 | 0.6703339 | 0.784151 | 0.167639154 | 0.6857703 | 0.6569706 | 0.6324848 |
| TNFSF9 | 0.252237883 | 0.3209812 | 0.372021 | 0.3127687 | 0.025656909 | 0.0509946 | 0.1242128 | 0.1140006 |
| EN-RAGE | 0.14757282 | 0.6423914 | 0.8206689 | 0.9097788 | 0.284330787 | 0.6077588 | 0.4223433 | 0.1726965 |
| CD6 | 0.135421058 | 0.0778894 | 0.4186959 | 0.5904386 | 0.106905101 | 0.1188304 | 0 | 0 |
| OPK | 0.100246829 | -0.012811 | -0.0700251 | 0.0153319 | -0.094029767 | -0.2679722 | -0.1184526 | -0.1120397 |
| CCP1 | 0.0996901 | 0.2596611 | 0.3436741 | 0.2811336 | 0.03771365 | 0.1401035 | 0.1155028 | 0.0534607 |
| TGF-alpha | 0.088679385 | 0.5673863 | 0.3664967 | 0.8218246 | -0.08400394 | -0.2654445 | -0.3552295 | -0.1402311 |
| IL-12B | 0.058951215 | -0.1178254 | -0.1456366 | -0.0797034 | 0.073884893 | 0.0415381 | 0.1629166 | 0.2285146 |
| IL-1-beta | 0.046003177 | 0 | 0 | 0.3201091 | 0 | 0 | 0 | 0 |
| LAP TGF-beta | 0.003902261 | 0.1936111 | 0.348399 | 0.4357403 | -0.216643794 | 0.1535455 | 0.1297112 | 0.1900107 |
| IL-15RA | 0 | 0.2714201 | 0.1808329 | 0.2705136 | 0 | 0.342803 | 0.3804132 | 0.4225055 |
| IL3 | 0 | 0.1800296 | 0.1109210 | 0.1823193 | 0 | 0.1013308 | 0.0610523 | 0.0148005 |
| CASP-8 | -0.285534818 | 0.1924253 | 0.1137314 | 0.6928641 | -0.508463382 | -0.3940538 | -0.2206238 | 0.0265747 |
| IL6 | -0.002494253 | -0.4442764 | -0.448338 | -0.4666233 | 0.194910996 | -0.2862909 | -0.3108963 | -0.5211096 |
| CC11 | -0.06830332 | -0.7556457 | -0.7220942 | -0.3834269 | -0.40539523 | -1.017311 | -0.666249 | -0.8432414 |
| FGF-19 | -0.24234679 | -0.1919048 | -0.891552 | -0.8040405 | -0.010186499 | -1.2771609 | -1.1496877 | -0.4633167 |
| IL7 | -0.318817211 | -0.4176902 | -0.3757189 | -0.3866335 | -0.457761252 | -0.5112329 | -0.4848198 | -0.4848694 |
| SCF | -0.430821848 | -1.187621 | -1.0019215 | -1.1411753 | -0.319673428 | -1.0665473 | -1.2085839 | -1.709935 |
| FGF-21 | -0.537983099 | -0.673921 | -0.6735678 | -0.5518102 | -0.253490754 | -0.451449 | -0.4523869 | -0.4219209 |
| 4E-BP1 | -1.833261928 | -1.6533508 | -1.6083197 | -1.3535527 | -2.92163411 | -2.0471707 | -1.9371138 | -1.9035556 |
| IL4 | -2.246212629 | -4.7017729 | -4.8063826 | -4.8110259 | -2.89501082 | -4.8309738 | -5.1340737 | -5.4777738 |
| DNM1 | -0.029443204 | -0.0924257 | -0.0924257 | -0.0526336 | 0 | 0 | 0 | 0 |
| CC110 | -0.059830704 | -0.0386807 | -0.0386807 | -0.0207188 | -0.213024488 | -0.2139249 | -0.2068125 | -0.1179569 |
| ST1A1 | -0.136230209 | -0.0280883 | -0.0176083 | -0.0101251 | 0 | 0.0015858 | 0.0369969 | 0.1162453 |
| GFNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL-17C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL-17A | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC111 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ADN1 | 0 | 0 | 0 | 0.0199076 | 0 | 0 | 0 | 0 |
| IL-20RA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC19 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL-7RB | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL2 | 0 | 0 | 0 | 0.0170553 | 0 | 0 | 0 | 0 |
| TSLP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| SLAMF1 | 0 | 0 | 0 | 0.052861 | 0 | 0 | 0 | 0 |
| MCP-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| FGF-23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL-10RA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| FGF-5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| LIF-R | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC119 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL-22 RA1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Beta-NGF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL-24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ARTN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| TNF | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC16 | 0 | 0 | 0 | 0.0221779 | 0 | 0 | 0 | 0 |
| IL-20 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IFN-gamma | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NRTN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.0608119 |
| CC125 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC13 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| NT-3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| CC120 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| IL5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| TNFR | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Detected (NPX value > +/- 0.3 compared to medium control)
 Not detected

| Abbr | Cytokines | Adjusted P values* | | | | | | | | | | | | | | | | | |
|---------------|--|--------------------|--------|-------------|--------------|--------|--------------------------------------|--------|--------|---------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| | | low | high | low vs high | NPX Baseline | SD | Stimulation through Low affinity igf | | | Stimulation through High affinity igf | | | | | | | | | |
| | | | | | | 3 hr | p | 6 hr | p | 24 hr | p | 3 hr | p | 6 hr | p | 24 hr | p | | |
| IL13 | Interleukin-13 | 0.8861 | 0.0084 | 0.0002 | 0.0000 | | | | | | | 0.0131 | 0.0000 | 0.0613 | 0.0000 | 0.0148 | 0.0000 | | |
| IL8 | Interleukin-8 | 0.1300 | 0.0007 | 0.0003 | 3.2887 | 1.6203 | | | | | | 1.7200 | 0.0002 | 1.8746 | 0.0001 | 1.9646 | 0.0000 | | |
| ILF | Leukemia Inhibitory factor | 0.1821 | 0.0009 | 0.0004 | 7.8294 | 1.4526 | | 1.0745 | 0.0000 | 1.1170 | 0.0047 | | 2.4301 | 0.0000 | 2.4039 | 0.0000 | 2.6519 | 0.0000 | |
| OSM | Oncostatin-M | 0.1171 | 0.0004 | 0.0001 | 8.1101 | 0.7300 | | 0.6432 | 0.0001 | 0.6400 | 0.0001 | 0.7484 | 0.0000 | 1.0708 | 0.0000 | 1.0405 | 0.0000 | | |
| HGF | Hepatocyte growth factor | 0.1121 | 0.0000 | 0.0000 | 5.7780 | 1.1617 | | | | | | 0.6122 | 0.0015 | 0.6904 | 0.0007 | 0.9320 | 0.0004 | 0.9200 | 0.0000 |
| CCL-2 | CCL-2, Macrophage chemoattractant protein 1 | 0.2473 | 0.0004 | 0.0001 | 8.3400 | 0.7964 | | 0.6125 | 0.0018 | | | | 1.0690 | 0.0001 | 1.1460 | 0.0000 | 0.9790 | 0.0000 | |
| CCL-3 | CCL-3, Macrophage inflammatory protein 1-alpha | 0.0007 | 0.0000 | 0.0000 | 3.8989 | 1.3780 | | 2.4000 | 0.0001 | 2.8024 | 0.0003 | 1.7788 | 0.0042 | 1.1400 | 0.0000 | 1.0621 | 0.0041 | | |
| CCL-4 | CCL-4, Macrophage inflammatory protein 1-beta | 0.0006 | 0.0000 | 0.0000 | 1.4571 | 0.8730 | | 1.8416 | 0.0000 | 1.2401 | 0.0000 | 1.0680 | 0.0006 | 1.0001 | 0.0000 | 1.0215 | 0.0000 | | |
| TGF- α | Transforming growth factor alpha | 0.2096 | 0.0012 | 0.0000 | 0.0000 | 1.0004 | | | | | | 0.7181 | 0.0188 | | | | | | |
| CCL3/4/MIP-1 | Macrophage chemoattractant protein 3 | 0.0006 | 0.0000 | 0.0000 | 0.0000 | 0.0000 | | | | | | | | | | | 1.0890 | 0.0000 | |
| SLAMF4 | CD244 | 0.2534 | 0.5506 | 0.0000 | 1.8017 | 2.0000 | | | | | | | | 0.9443 | 0.0000 | | | | |
| Abbr | Cytoplasmic molecules | low | high | low vs high | NPX Baseline | SD | 3 hr | p | 6 hr | p | 24 hr | p | 3 hr | p | 6 hr | p | 24 hr | p | |
| CASP-8 | Caspase 8 | 0.1003 | 0.1089 | 0.1140 | -0.3970 | 0.8730 | | 0.4780 | 0.0077 | 0.5991 | 0.0036 | 0.9784 | 0.0000 | | | | 0.4813 | 0.0188 | |
| SIRT2 | SIRT-like protein 2 | 0.0407 | 0.0010 | 0.0001 | 0.2309 | 0.2000 | | | | 0.1987 | 0.0007 | 0.4006 | 0.0041 | | | | | | |
| STAMP | STAM-binding protein | 0.1121 | 0.0110 | 0.4440 | 0.2203 | 0.1975 | | | | 0.1793 | 0.0116 | 0.4912 | 0.0003 | 0.5181 | 0.0041 | 0.4893 | 0.0007 | 0.4648 | 0.0000 |
| Abbr | Type II TM molecules | low | high | low vs high | NPX Baseline | SD | 3 hr | p | 6 hr | p | 24 hr | p | 3 hr | p | 6 hr | p | 24 hr | p | |
| CD40 | CD40 | 0.0200 | 0.4403 | 0.0000 | 0.0000 | 1.6170 | | | | 2.1177 | 0.0012 | 1.1725 | 0.0009 | | | | | | |
| CD8 | T-cell surface glycoprotein CD8 | 0.0007 | 0.1079 | 0.0000 | 0.0000 | 0.7000 | | | | 1.2000 | 0.0000 | 0.3774 | 0.0000 | | | | | | |
| IL10RB (IL10) | Interleukin-10 receptor subunit beta | 0.1821 | 0.0009 | 0.0000 | 0.4017 | 0.5676 | | | | | | 0.1717 | 0.0044 | | | | | | |
| CD8 | T-cell surface glycoprotein CD8 isoform | 0.0704 | 0.0010 | 0.0000 | 0.1312 | 0.2076 | | | | | | 0.4910 | 0.0112 | | | | | | |
| PH-1 | CD274, Programmed cell death ligand 1 | 0.0708 | 0.0104 | 0.0004 | 2.8377 | 0.3742 | | | | 0.8382 | 0.0010 | 0.9424 | 0.0003 | 0.8225 | 0.0012 | 0.9185 | 0.0004 | 1.0103 | 0.0001 |
| IL18 | IL18 | 0.1821 | 0.0000 | 0.0000 | 2.0004 | 0.0000 | | | | | | | | | | | | | |
| CSF-1 | Macrophage colony-stimulating factor 1 | 0.1821 | 0.0000 | 0.0000 | 2.4402 | 0.9683 | | | | | | 0.6347 | 0.0100 | 0.6009 | 0.0078 | 0.8834 | 0.0000 | 1.2389 | 0.0000 |
| PLTL | Fms-related tyrosine kinase 3 ligand | 0.1495 | 0.0007 | 0.2195 | 3.4640 | 0.7097 | | | | | | 0.5603 | 0.0009 | | | 0.6000 | 0.0001 | 0.6084 | 0.0001 |
| Abbr | Type II TM molecules | low | high | low vs high | NPX Baseline | SD | 3 hr | p | 6 hr | p | 24 hr | p | 3 hr | p | 6 hr | p | 24 hr | p | |
| TWEAK | TWEAK, TNFSF12 | 0.2096 | 0.0010 | 0.0000 | 5.1847 | 1.3600 | | | | 1.2984 | 0.0000 | 1.2465 | 0.0001 | 1.0513 | 0.0000 | 1.0490 | 0.0000 | | |
| LIGHT | LIGHT, TNFSF14, CD256 | 0.1121 | 0.0000 | 0.0724 | 1.8516 | 1.3792 | | | | 1.0725 | 0.0009 | 0.0914 | 0.0114 | 1.4640 | 0.0000 | 1.6134 | 0.0000 | 1.8742 | 0.0000 |

*One p-value to determine if differences over time are observed (is there any differences in expression over time (low, high or between low and high affinity activation))
 **specific p-value for each time point (at what time point is a significant difference observed)
 *** igf/iC compared to resting state