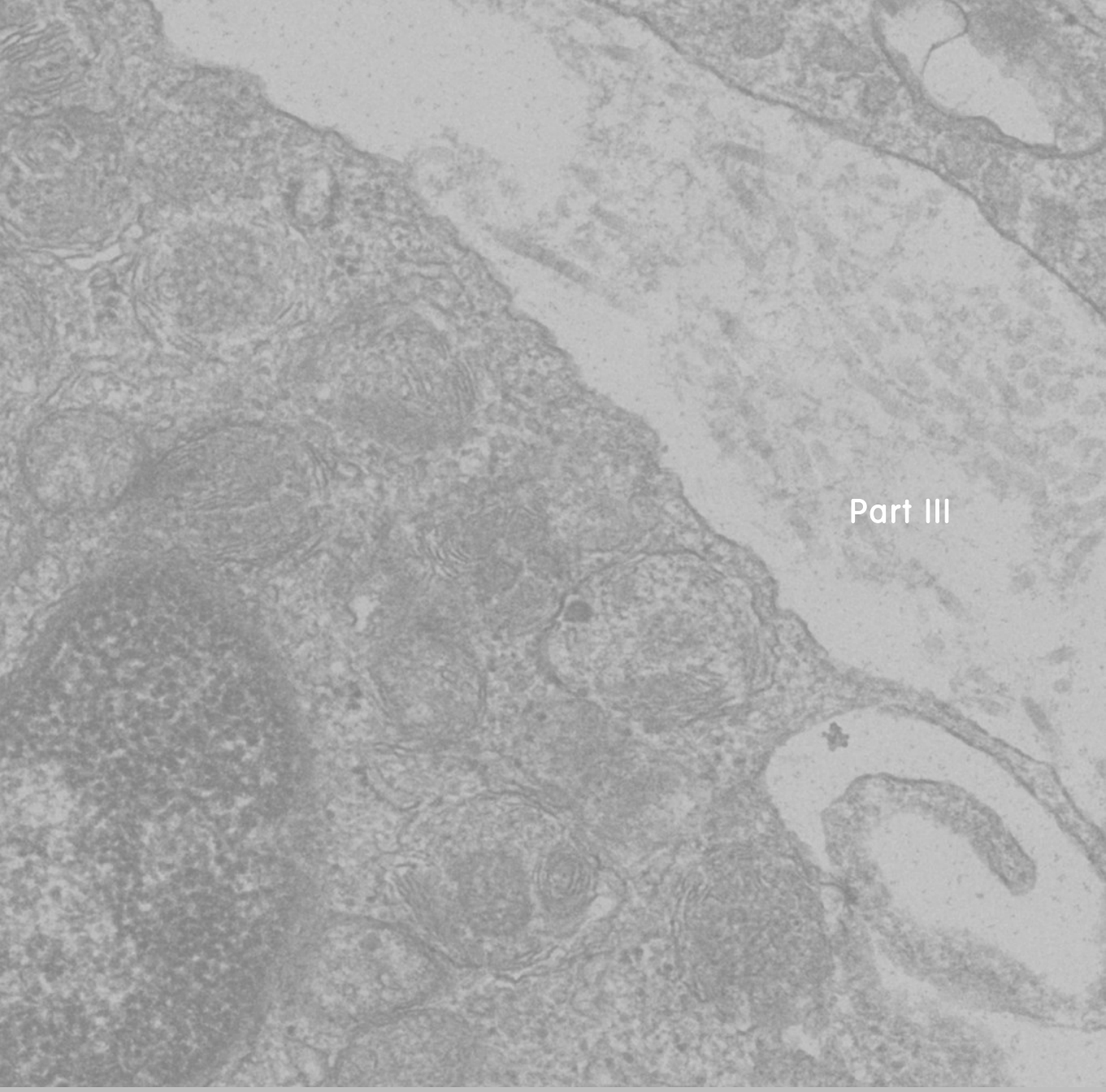

Part III

Antibody-mediated chronic inflammation in allergy and autoimmunity

REPEATED FCεRI TRIGGERING REVEALS MODIFIED MAST CELL FUNCTION RELATED TO CHRONIC ALLERGIC RESPONSES IN TISSUE

Chapter 10

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ABSTRACT

Background Activation of mast cells through FcεRI plays an important role in acute allergic reactions. However, little is known about the function of mast cells in chronic allergic inflammation, or the impact of repeated FcεRI triggering occurring in such responses.

Objective We aimed to identify changes in mast cell function after repeated FcεRI triggering, and to correlate these changes to chronic allergic responses in tissue.

Methods Human cord blood-derived mast cells were treated for two weeks with anti-IgE. The function of naive or treated mast cells was analysed by RNA sequencing, quantitative RT-PCR, flow cytometry, and functional assays. Protein secretion was measured using ELISA and multiplex assays.

Results We observed several changes in mast cell function after repeated anti-IgE triggering. Whereas the acute response was dampened, we identified 289 genes significantly upregulated after repeated anti-IgE. Most of these genes (84%) were not upregulated after a single anti-IgE stimulus indicating a significantly different response mode, characterized by increased antigen presentation, response to bacteria, and chemotaxis. Changes in the mast cell function were related to changes in expression of transcription factors RXRA and BATF and others. Importantly, we found a substantial overlap between the genes upregulated after repeated anti-IgE triggering with genes upregulated in chronic allergic tissues, in particular chronic rhinosinusitis.

Conclusion Our study provides evidence for intrinsic modulation of mast cell function upon repeated FcεRI-mediated activation. The overlap with gene expression in tissues is suggestive of a direct link between repeated IgE-mediated activation of mast cells and chronic allergy.

KEY MESSAGES

Repeated activation of mast cells through FcεRI leads to a modulation of their function, related to changes in gene expression during chronic allergy

Chronic activation of mast cells such as occurring in chronic allergy may therefore contribute to changes in tissue homeostasis

INTRODUCTION

Mast cells play an important role in allergic responses upon activation through the high-affinity FcεRI. Their rapid degranulation and release of lipid-derived mediators leads to an immediate response, characterized by increased vascular permeability. This response can also lead to several tissue-specific phenomena, such as contraction of airway smooth muscle cells and mucus secretion in the airways, or anaphylaxis in case a systemic response is triggered (*1, 2*). Acute symptoms can occur within minutes of exposure to the associated allergens, and are thought to be induced through release of histamine, prostaglandins and leukotrienes by mast cells. During late phase responses, mast cell-derived cytokines and chemokines (IL-5, IL-13) also contribute to the inflammatory reactions observed in allergy, in particular through their effects on leukocyte infiltration and activation (*3*).

However, allergic disease is often associated with repeated exposure to allergens which is thought to lead to chronic inflammation and changes in tissue homeostasis. In the lungs, these may lead to increased contractability of the smooth muscles, leading to obstruction of the bronchia, increased mucus production, and airway wall remodeling (*1*). In the skin, atopic dermatitis is characterized by fibrotic lesions, containing thickened dermis and epidermis, and accumulation of lymphocytes and mast cells (*4, 5*). Chronic rhinosinusitis (CRS) is characterized by inflammation of the upper airways. CRS is frequently divided into two groups based on the presence of nasal polyps. CRS with nasal polyps is associated with nasal obstruction and olfactory loss and is characterized by eosinophilia and Th2-related inflammation (*6, 7*).

Importantly, these diseases are all associated with repeated antigen exposure and triggering through IgE. Whereas mast cells and IgE antibodies play a crucial role in acute symptoms of an allergic reaction, their role in chronic inflammation and tissue remodeling is less well-defined (*8*). Chronic allergic responses in tissue are the result of a complex interplay between different immune cells (mast cells, eosinophils, macrophages, dendritic cells, lymphocytes) and stromal cells. Mast cells can secrete several mediators that have the potential to influence tissue remodeling, such as FGF-2, VEGF and amphiregulin (*9, 10*).

In some mouse models of chronic asthma, mast cells are dispensable for induction of eosinophilia or airway hyperresponsiveness. These models are thought to be driven by the Th2-derived cytokines IL-4 and IL-5 (*11-14*). However, in other mouse models of chronic asthma, induced through repeated antigen exposure after initial sensitization, mast cells and activation through FcεRI are shown to be crucial for several hallmarks of chronic asthma, such as tissue remodeling, goblet hyperplasia and leukocyte infiltration (*15-17*). Mast cells have also been shown to play a crucial role in chronic dermatitis in mice (*5, 18*).

However, the exact changes in mast cells that occur after repeated antigen exposure are not well understood, especially in the context of human disease. Although mast cells alone are unlikely to provide all the signals leading to tissue remodeling, appreciation of the mechanisms contributing to their involvement in chronic inflammation can enhance our understanding of these diseases and thereby allow us to design novel therapeutics aimed at the long-term changes induced in tissues.

As tissue-resident mast cells are long-lived cells (estimated up to months or years) (*19*), we hypothesized that mast cells will be exposed repeatedly to antigen, and that this may lead to intrinsic changes in mast cell function and/or biology relevant to chronic allergy. Studies using several pre-clinical (asthma) animal models have offered significant progress in our understanding of the role and function mast cell function in (chronic) allergy. Nonetheless, still little is known on cell-intrinsic changes induced upon repeated antigen exposure of mast cells in general and human mast cells in particular.

In this study, we modelled repeated antigen exposure of human mast cells in vitro, and show that mast cells respond differently after repeated- compared to acute FcεRI activation. We captured these changes in mast cell function using full RNA sequencing, and identified several novel genes specifically upregulated after repeated activation. Furthermore, the expression pattern of these genes related to changes in gene expression also observed in tissue during chronic atopic diseases, together indicating that repeated antigen exposure of mast cells triggers a response mode that may directly contribute to tissue remodeling and inflammation.

METHODS

EXPERIMENTAL SETUP

Cord blood-derived mast cells were obtained as described in Supplementary Methods. The purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI, CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99% (Supplementary Fig 1 A-D). Cultured mast cells were sensitized with 1 µg/mL hybridoma IgE (Diatec) for a minimum of 24 hours before each stimulation. Mast cells were cultured at $1x10^6$ cells/mL.

For repeated anti-IgE stimulations, mast cells were stimulated once weekly with 0.2 µg/mL goat-anti-human IgE (Nordic). After two weeks, mast cells were counted using Trypan Blue, and cultured again at $1x10^6$ cells/mL. Mast cells were stimulated with control medium or 1 µg/mL anti-IgE for 6 hours for RNA analysis, or with 0.01-10

µg/mL anti-IgE for 1, 6 and 24 hours for analysis of protein expression and secretion. For supernatant transfer experiments, sensitized mast cells were stimulated with 0.2 µg/mL goat-anti-human IgE (Nordic) or control medium. After 24h, supernatant was harvested. Non-sensitized mast cells were cultured in this supernatant for two weeks. Supernatant was replaced after 1 week. At the end of two weeks, mast cells were sensitized and activated as described above for collection of RNA, and analysis of protein expression and cytokine secretion.

RNA isolation, sequencing, qPCR, measurements of protein expression measurements and comparisons to publicly available gene expression data were performed as described in Supplementary methods, and samples processed for RNA-seq are shown in Online Repository Table E2.

STATISTICAL ANALYSIS

For comparison of naïve and treated mast cells, paired-samples T-test was performed. For differences between multiple groups, repeated measures ANOVA was performed, with Bonferroni's post-hoc test to correct for multiple testing. To compare frequencies of gene overlap, Fisher's Exact test was used. P values of <0.05 were considered statistically significant (except for the sequencing analysis, where significance was defined as described above). Statistical analysis was performed using GraphPad Prism 5. Venn diagrams were created using BioVenn (*20*).

RESULTS

REPEATED FCεRI TRIGGERING OF MAST CELLS

We set up a model for repeated FcεRI triggering of mast cells to mimic repeated IgE-mediated activation such as occurring during chronic allergic responses (Fig 1A). Human cord blood-derived mast cells were stimulated for two weeks with 0.2 µg/mL anti-IgE (Repeated αIgE) or control medium (Naive). We chose to stimulate the cells weekly, as more frequent stimulations with anti-IgE led to decreased FcεRI expression, resulting from prior stimulations (data not shown). The first stimulation led to a moderate level of degranulation of mast cells as well as cytokine production (Fig 1B,C). Twenty-four hours prior to each stimulation, mast cells were sensitized with human IgE. At the end of the two weeks, mast cells were either left untreated (N and R) or stimulated with anti-IgE (N+αIgE and R+αIgE).

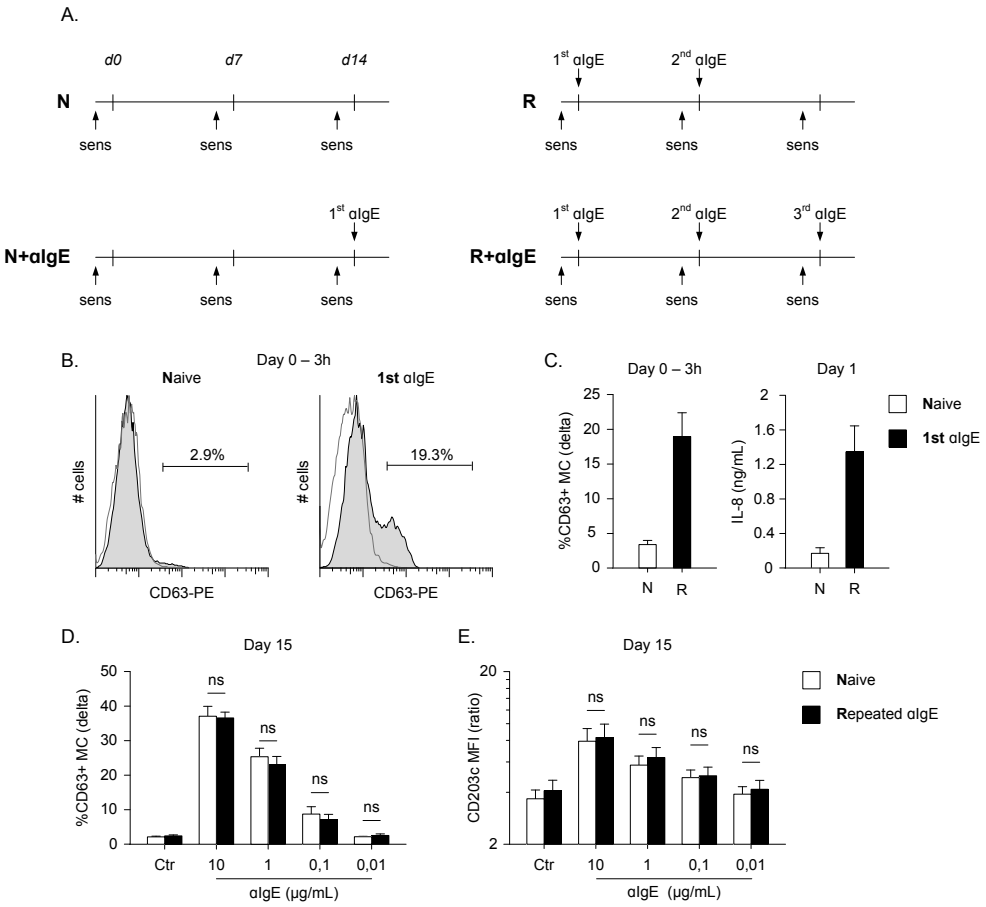

Figure 1. Repeated mast cell stimulation through FcεRI. A) Experimental setup of repeated stimulation of mast cells. Human cord blood-derived mast cells were stimulated for two weeks with 0.2 μg/mL anti-IgE (Repeated αIgE) or control medium (Naive). Twenty-four hours prior to each stimulation, mast cells were sensitized with human IgE. At the end of the two weeks, mast cells were either left untreated (N and R) or stimulated with anti-IgE (N+αIgE and R+αIgE). B) Representative flow cytometry plots for CD63 of naive mast cells (N) or mast cells 3 hours after their first anti-IgE stimulation (R), showing their degranulation in response to the first anti-IgE on day 0. C) Summary of surface CD63 expression as in B) and IL-8 secretion 24 hours after the first anti-IgE stimulation or control medium. D, and E) Surface CD63 and CD203c expression in response to 24 hours of anti-IgE stimulation, in naive mast cells or mast cells exposed to two weeks of stimulation (Repeated αIgE). CD63 staining as in C, and CD203c MFI ratio calculated as in E. Data are represented as mean ± SEM from n=3 (C) or n=6 (D, and E) independent experiments. Ns indicates that no significant differences were found between naive mast cells (N) and cells exposed for two weeks to anti-IgE (R), using paired T-test.

No difference in cell number, viability, maturation, or morphology was observed between the different treatments (Supplementary Fig 1 E-I). After two weeks, mast cells were stimulated with anti-IgE to evaluate changes in mast cell function when they had been previously exposed to anti-IgE. No difference in degranulation (CD63 upregulation) or upregulation of activation marker CD203c was observed after repeated activation through FcεRI (Fig 1D, E).These results show that in our in vitro model, mast cells maintained the intrinsic capacity to respond to FcεRI triggering after repeated activation through this receptor, allowing the study of downstream functional changes in these cells after (repeated) stimulation of the FcεRI-receptor.

DISTINCT RESPONSES TO FCεRI TRIGGERING UPON REPEATED EXPOSURE

To capture the complete mast cell transcriptome after two weeks of stimulation through FcεRI, we performed deep sequencing on RNA from three independent experiments. We isolated RNA 6 hours after stimulation with control medium or anti-IgE at the end of the two weeks treatment. Quality controls are described in the Supplementary Methods, and results are shown in Online Repository Table E2 and Supplementary Figure 2.

To analyse the response of mast cells after repeated anti-IgE stimulation, we first selected those genes that are upregulated by single anti-IgE stimulation in naïve mast cells (Fig 2A, Online Repository Table E4A) (*21*). Expression of 1919 genes (449 genes >2-fold) was significantly upregulated after 6 hours of single stimulation with anti-IgE. The average upregulation of these genes was significantly dampened in mast cells which were exposed repeatedly to anti-IgE for 2 weeks (R+αIgE) (Fig 2B,C). Of these 449 genes, 22 showed a significant dampening of their expression after repeated stimulation (Online Repository Table E5A, E6A, and Fig 2D). CSF2 and CCL4, cytokines/chemokines known to be secreted by mast cells, displayed the most pronounced dampened responses. We validated these findings by qPCR and measured secreted proteins GM-CSF (CSF2) and MIP-1β (CCL4) in mast cell supernatant obtained after 24h (Fig 2E-J) confirming this dampened response at the protein level in both GM-CSF and CCL4 (albeit a non-significant trend for CCL4). These results suggest a reduced responsiveness to FcεRI triggering by mast cells after repeated stimulation. In support of this notion, the expression of those genes that were downregulated by a single anti-IgE stimulation, was less downregulated after repeated stimulation (Supplementary Fig 3, Online Repository Table E4B), suggesting an overall dampening of mast cell transcriptional responses to FcεRI triggering after repeated stimulation.

We did not observe changes in expression of most genes associated with arachidonic acid-derived products (Fig 2K and Online Repository Table E7). In contrast to the decreased production of cytokines we observed, degranulation and release of lipid mediators LTC_4 and

PGD_2 were not affected by repeated FcεRI triggering (Fig 1H and Fig 2L,M). These results suggest that repeated FcεRI triggering affected the *de novo* transcription of FcεRI-responsive genes, but not initial FcεRI signaling leading to degranulation and release of lipid mediators.

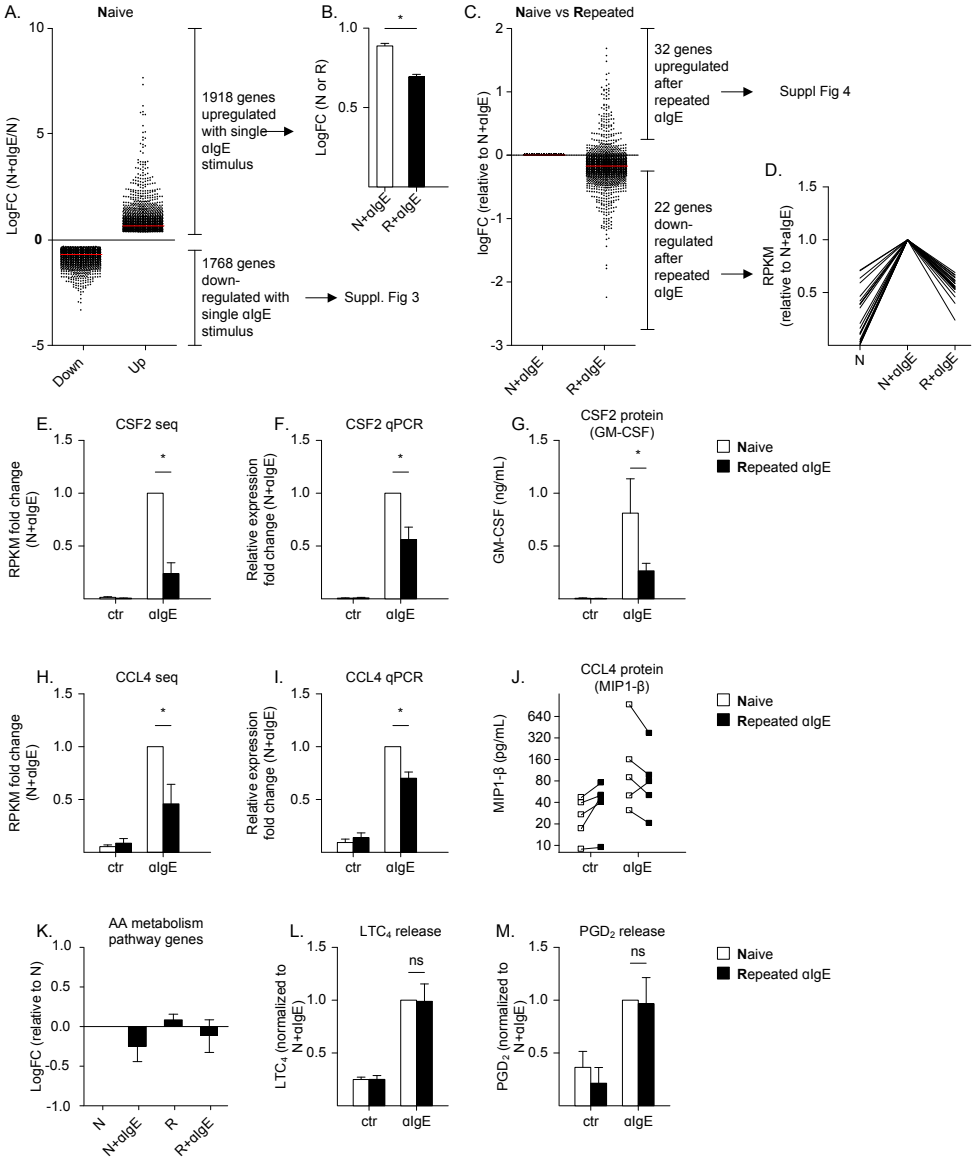

Figure 2 *(left)*. Dampened genes after repeated anti-IgE. A) Gene expression obtained using RNA sequencing in response to a single 6 hour anti-IgE stimulation (N+αIgE), compared to control mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. B) Genes which were significantly upregulated after a single anti-IgE were selected as shown in A), and compared to their upregulation after repeated stimulation (R+αIgE). LogFC was calculated compared to unstimulated mast cells at the end of the two week stimulation (N+αIgE/N and R+αIgE/R). C) Genes which were significantly upregulated after a single anti-IgE were selected as shown in A), where each dot indicates a different gene. Those genes significantly upregulated (top) and tolerized (bottom) after repeated anti-IgE (R+αIgE) compared to a single anti-IgE (N+αIgE) stimulation are indicated. Data are shown as fold change relative to N+αIgE. D) Genes which are significantly downregulated after repeated anti-IgE, compared to single anti-IgE using differential expression analysis. Each line represents a single gene, and data is shown as relative RPKM normalized to single anti-IgE stimulation (N+αIgE). E, and H) Gene expression of CSF2 and CCL4, obtained using RNA sequencing. Data is shown as RPKM fold change relative to single anti-IgE stimulation (N+αIgE). F, and I) Gene expression of CSF2 and CCL4, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to single anti-IgE stimulation (N+αIgE). G, and J) Protein levels of CSF2 (GM-CSF) and CCL4 (MIP-1β) in supernatant, 24 hours after stimulation with anti-IgE of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. K) Expression of genes in the Arachidonic Acid (AA) metabolism pathway, obtained using RNA sequencing in response to a single 6 hour anti-IgE stimulation (N+αIgE), compared to control mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. L,M) Protein levels of LTC4 and PGD2 in supernatant, 1 hour after stimulation with anti-IgE of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. Data are represented as mean ± SEM from n=3 (B, E, H, and K) or n=5 (F, G, I, and J) or n=6 (L and M) independent experiments. Asterisks indicate significant differences obtained using paired T-test $p<0.05$ (B), differential expression analysis FDR<0.05 (C, E, and H) or repeated measures ANOVA with Bonferroni posthoc test $p<0.05$ (F, G, I, L, and M).

Despite this dampening of the acute response of mast cells on the global level, we observed that 32 FcεRI-responsive genes displayed increased upregulation of their expression after repeated stimulation with anti-IgE (Fig 2C, Supplementary Fig 4A, Table E5B, Online Repository Table E6B). The upregulation of the top three significant genes, TMEM45B, EMR3 and CH25H was confirmed using qPCR (Supplementary Fig 4B-G).

Together, these results show that repeated anti-IgE triggering alters the mast cell response, characterized by overall dampening of most FcεRI-responsive genes, as well as by upregulation of a particular set of genes.

To capture whether there were functional differences between the genes which were dampened compared to those that showed further upregulation, we analysed GO biological pathways represented by these genes (Online Repository Table E8).

Dampened responses were assigned to pathways involved in leukocyte proliferation and activation (6 out of 22 genes), whereas upregulated genes participated mainly in pathways involved in the response to bacteria (5 out of 32 genes), response to wounding (6 out of 32 genes), and chemotaxis (3 out of 32 genes).

Together, these results show that mast cell function can be modulated by repeated FcεRI triggering resulting in a different response mode as compared to acute stimulations.

DE NOVO UPREGULATION UPON REPEATED FCεRI TRIGGERING

To capture any other changes occurring in the mast cell transcriptome after repeated stimulation with anti-IgE, irrespective of their responsiveness to single anti-IgE stimulation, we performed differential expression analysis on the total gene set. Two hundred eighty nine (289) genes were significantly upregulated after repeated anti-IgE stimulation, of which 243 genes did not respond to single anti-IgE stimulation (Online Repository Table E9). Upregulation of most of these genes did not increase further by 6 hours of the additional 3rd anti-IgE stimulation (R+αIgE), consistent with a persistent upregulated expression (Fig 3A,B). The 20 most strongly upregulated genes are shown in Online Repository Table E5C. Upregulation of TREM2, OLFM4 and CCL18 were confirmed using qPCR (Fig 3C-H).

To verify the upregulation of the most highly upregulated gene CCL18 at the protein level, the secretion of CCL18 was measured in supernatant. Whereas secretion of CCL18 secretion was not detected upon acute anti-IgE activation of naive mast cells stimulated for 6 or 24 hours, we observed a significant release of CCL18 after 2 weeks of stimulation through FcεRI (Fig 3I,J), confirming the sustained secretion of CCL18 in supernatant after repeated stimulation with anti-IgE.

As a control for the specificity of anti-IgE stimulation, we have stimulated mast cells with IgG immune complexes at the end of the two weeks culture with anti-IgE. In contrast to restimulation with anti-IgE, cytokine production upon stimulation with platebound IgG immunecomplexes was not affected or was even increased (CCL4) when mast cells had been treated for two weeks with anti-IgE (Supplementary Fig 5A-C). Furthermore, stimulation of mast cells through FcγRIIA for two weeks did not affect gene expression of CSF2, CCL4 and CCL18 (Supplementary Fig 5 D-F).

These data further confirm the specificity of FcεRI stimulation, and suggest that the changes in mast cell function as observed after repeated stimulation are FcεRI-intrinsic.

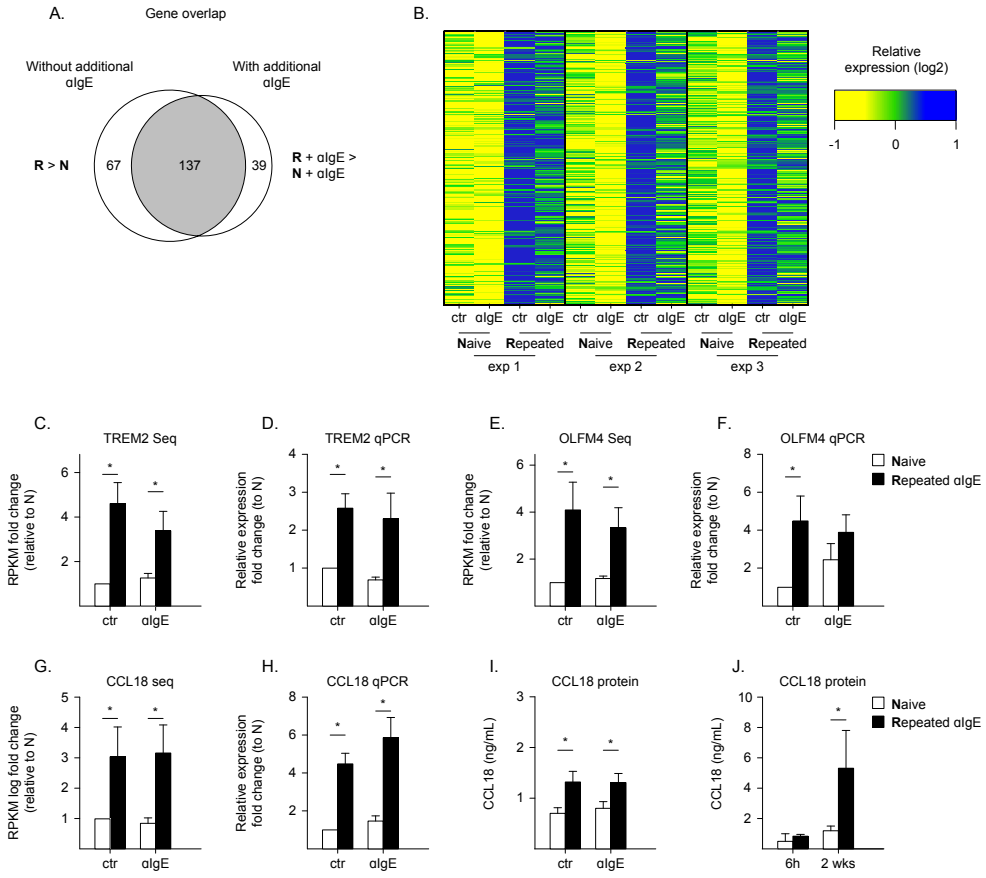

Figure 3. Persistent upregulation of genes after repeated anti-IgE. A) Overlap of genes upregulated after repeated anti-IgE, without an additional stimulation (R>N) or with an additional stimulation (R+αIgE>N+αIgE), obtained using differential expression analysis. B) Heatmap showing relative expression of all genes in A. Each row represents a single gene. The relative expression in 3 independent experiments is shown. C, E, and G) Gene expression of TREM2, OLFM4, and CCL18, obtained using RNA sequencing. Data is shown as RPKM fold change relative to naïve mast cells (N). D, F, and H) Gene expression of TREM2, OLFM4, and CCL18, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to naïve mast cells (N). I) Protein levels of CCL18 in supernatant, 24 hours after stimulation with anti-IgE of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. J) Protein levels of CCL18 in supernatant, 6 hours after stimulation with anti-IgE of naive mast cells (left), or 2 weeks after stimulation with repeated anti-IgE or control medium (right). Data are represented as mean ± SEM from n=3 (C, E, G, and J) or n=5 (D,F, and H,) or n=4 (I) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (C, E, and G), repeated measures ANOVA with Bonferroni posthoc test $p<0.05$ (D,F, H, and I), or paired T-test $p<0.05$ (J). N = naive mast cells; N+αIgE = a single 6 hours stimulation of naïve mast cells with anti-IgE; R = repeated anti-IgE stimulation for two weeks; R+αIgE = 6 hours stimulation with anti-IgE after repeated anti-IgE stimulation.

MODULATION OF MAST CELL FUNCTION REVEALED THROUGH PATHWAY ANALYSIS

To confirm that the genes upregulated after repeated FcεRI triggering were not found after single anti-IgE stimulation, we compared them to those genes upregulated after a single anti-IgE stimulus in the CAGE study (*22*) (Supplementary Fig 6A,B). Only a small proportion of these genes overlapped (less than 10%), confirming that the repeated stimulation through FcεRI leads to changes in mast cell function, characterized by upregulation of novel genes. To analyse whether upregulated expression of these novel genes after repeated FcεRI stimulation is related to global functional changes, we performed pathway enrichment analysis of these genes, using GO term biological pathways in DAVID (Online Repository Table E10) (*23*).

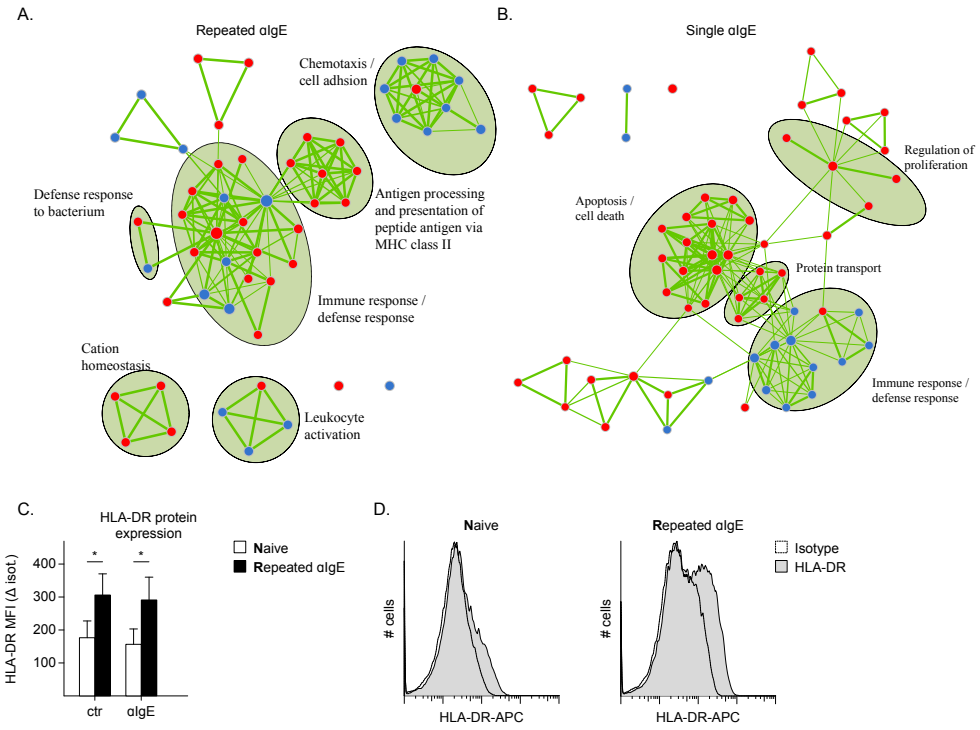

Figure 4. Overlap in genes and enriched pathways between repeated and single stimulation of mast cells. A, and B) Interaction network of GO terms enriched in genes upregulated after repeated anti-IgE (A) or single anti-IgE (B) stimulation. Node size reflects the significant/number of genes, and thickness of the lines indicates the connectivity between two GO terms. Pathways that are unique for either of the gene sets are highlighted in red. C, and D) Protein expression of HLA-DR, obtained using flow cytometry. Data is shown as delta MFI of HLA-DR compared to isotype control. Data are represented as mean ± SEM from n=5 (C) independent experiments. Asterisks indicate significant differences obtained using repeated measures ANOVA with Bonferroni posthoc test $p<0.05$ (C).

Although a substantial number of pathways overlapped, the majority (60-70%) of enriched pathways was uniquely enriched after repeated stimulation compared to single stimulation (Supplementary Fig 6 C,D). Biological pathways were then clustered using Cytoscape and DAVID, a clustering analysis based on the overlap of genes in closely related biological pathways. (Fig. 4A,B, Online Repository Table E10) (*24, 25*). The most prominent pathway clusters after repeated FcεRI triggering were "antigen processing and presentation", "response to bacteria", and "chemotaxis". To confirm the upregulation of key genes involved in antigen presentation, we analysed the expression of HLA class II genes by qPCR, confirming the sequencing results (Supplementary Fig 7). Furthermore, protein expression of HLA-DR, analysed using flow cytometry showed significantly increased cell surface expression of HLA class II (Fig 4C,D).

Since we observed significant pathways related to "response to bacteria" upon repeated triggering, we next sought out to confirm this enhanced response to bacteria in our in-vitro setup, using LPS to stimulate TLR-4 (Supplementary Fig 8A-C, Online Repository Table E11). A significant enhancement in upregulation of LPS-responsive genes was observed. Furthermore, the RNA expression and protein secretion of LPS-induced cytokines IL-8 and CCL4 were also significantly enhanced after repeated activation through FcεRI (Supplementary Fig 8D-I).

Together, these results show that mast cell function after repeated anti-IgE exposure is modulated, and enriched for a variety of pathways including antigen processing and presentation, response to bacteria, and chemotaxis.

MODULATION OF MAST CELL TRANSCRIPTOME RELATED TO CHANGES IN TRANSCRIPTION FACTORS RXRA AND BATF

To analyse whether autocrine signaling of factors released by mast cells themselves could contribute to the changes in gene expression, we studied whether supernatant from activated mast cells could induce similar gene expression profiles as direct stimulation. Downregulation of CSF2 and CCL4 and upregulation of EMR3 were only induced by direct stimulation (Fig 5A-C). In contrast, upregulation of TMEM45B, TREM2, and CCL18 were also induced by supernatant transfer suggesting a role for autocrine signaling in the upregulation of these genes (Fig 5D-F). qPCR data for CSF2, CCL4 and CCL18 were confirmed on the protein level by ELISA (data not shown).

These results suggest that the modulation of expression of some genes can be explained through autocrine signaling, although the expression of other genes is directly affected by repeated FcεRI-signaling.

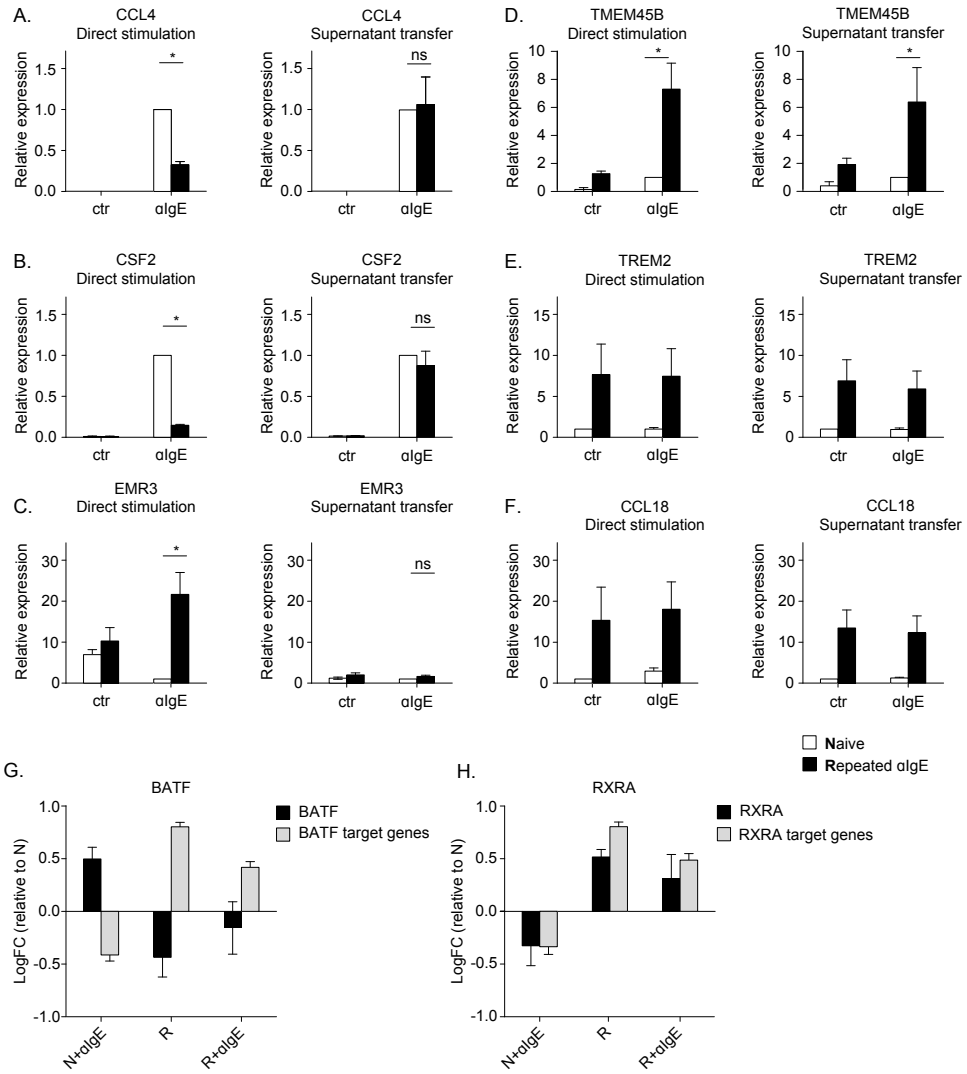

Figure 5. Autocrine signaling and transcription factors. A-F) Mast cells were stimulated for two weeks with 0.2 µg/mL anti-IgE (Repeated αIgE) or control medium (Naive) through direct stimulation (left) or using supernatant from activated mast cells obtained 24h after activation (right). Gene expression of CCL4, CSF2, EMR3, TMEM45B, TREM2, and CCL18, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to cells stimulated for 6h with anti-IgE (CCL4, CSF2, EMR3, TMEM45B) or to naïve mast cells (TREM2, CCL18). G-H) RNA expression analysis of transcription factors BATF (A) and RXRA (B) and their target genes as identified by Cscan enrichment analysis. Expression of these genes in mast cells exposed to repeated anti-IgE stimulation is shown as fold change relative to naive mast cells. Data in are represented as mean ± SEM from n=4 independent experiments (A-F) or from n=3 independent experiments (G-H). Asterisks indicate significant differences obtained using or repeated measures ANOVA with Bonferroni posthoc test $p<0.05$.

To analyse whether transcription factors are involved in the regulation of transcription after repeated activation of mast cells through FcεRI, we first analysed the presence of transcription factors in our differentially expressed genes using DAVID (Online Repository Table E12). We observed upregulated expression of BHLHE41, CEBPA, CEBPD, CEBPE, KLF2, MAFB, NFE2, RXRA, STAT4, and downregulated expression of BATF and NR4A1. In order to identify whether target genes of these transcription factors were significantly enriched in the differentially expressed genes after repeated FcεRI triggering, we used the analysis tools Cscan and Pscan to assesses which transcription factor binding sites are significantly overrepresented, providing thus hints on which transcription factors could be common regulators of these genes (Online Repository Table E13) (*26, 27*).

Significantly enrichment for binding sites or predicted binding affinity to CEBPA, RXRA, BATF was observed in at least two of the analysis tools used. Interestingly, one of the transcription factors that showed decreased expression after repeated FcεRI stimulation, BATF, is known to be a transcriptional repressor (*28*). Indeed, when we analysed the gene expression profiles of target genes for these transcription factors as derived from Cscan analysis, there was an inverse relationship between expression of BATF and its target genes (n=73), whereas there was a positive relationship between RXRA (transcriptional activator) with its target genes (n=60) (Figure 5G,H and Online Repository Table E14). Together, these two transcription factors were found to bind the regulatory regions of 101 of the 218 de novo upregulated genes which were analysed in Cscan. Because Pscan does not use experiment-derived data, and CEBPA was only present in the Pscan analysis tool, we could not study the expression of CEBPA target genes.

Together, these results indicate that changes in expression of transcription factors RXRA, BATF and possibly others, including CEBPA, may contribute to the modified RNA expression profile after repeated FcεRI stimulation.

NOVEL GENES UPON REPEATED FCεRI TRIGGERING ARE IMPLICATED IN CHRONIC ATOPIC TISSUES

As some of our most highly upregulated genes, such as CCL18, have been implicated in chronic atopic conditions (atopic nasal polyps, atopic asthma, and atopic dermatitis), we next analysed the overlap of our genes with genes upregulated in tissue of these chronic atopic conditions, identified using microarrays or RNA-seq (Online Repository Table E15). We found a striking overlap with genes associated with atopic allergies, in particular with tissue from nasal polyps from atopic rhinosinusitis patients (Fig 6A,B and Supplementary Fig 9A,B).

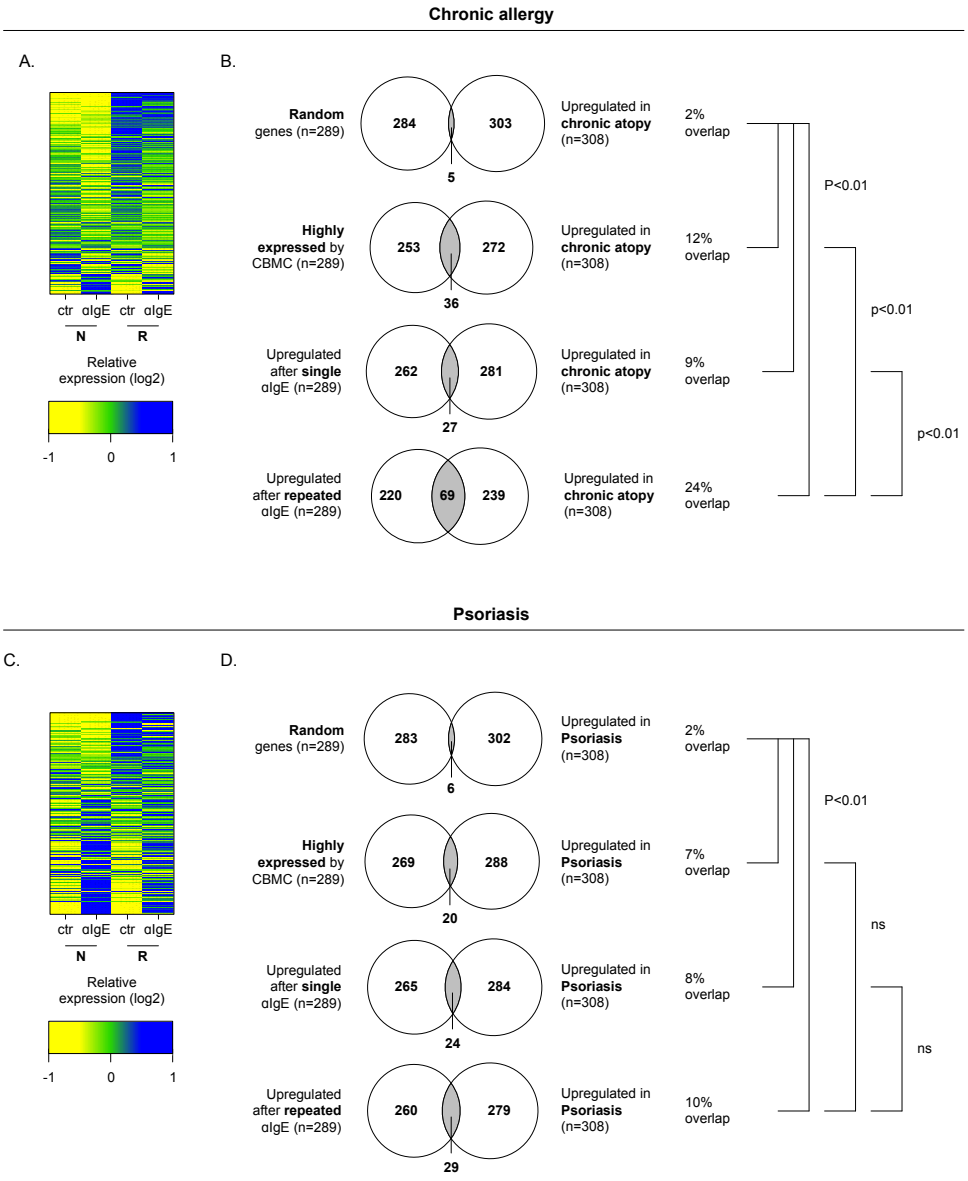

Figure 6. Association of upregulated genes with chronic allergy. A, C) Heatmap showing relative expression of genes upregulated in atopic allergy patients or psoriasis patients in mast cells exposed to repeated anti-IgE stimulation. B, D) Overlap of genes upregulated in atopic allergy patients or psoriasis patients with random genes (top), genes most highly expression by mast cells (2nd row), genes upregulated in mast cells after a single anti-IgE stimulus (3rd row), or genes upregulated in mast cells after repeated anti-IgE (bottom). Data are represented as mean ± SEM from n=3 (A and C) independent experiments. Overlap was calculated as percentage of the mast cell or random gene list. Asterisks indicate significant differences obtained using paired T-test $p<0.05$ (A and C), or Fisher's exact test $p<0.05$ (B and D).

Sixty-nine out of 308 genes upregulated in chronic allergy were significantly upregulated upon repeated stimulation of mast cells through FcεRI, representing 24% of the upregulated genes after repeated stimulation (Fig 6B), a percentage much higher than that with random genes (max 2%), the genes most highly expressed by mast cells (12%), or the genes upregulated with a single anti-IgE stimulus (9%). The overlap with two other atopic diseases was less pronounced, but for atopic dermatitis still more than obtained with random genes (Supplementary Fig 9C,D).

As control, the same analysis was performed with genes upregulated in psoriasis (Fig 6C,D). Whereas psoriasis genes were related to genes highly expressed by mast cells, possibly reflecting the increased number of mast cells in psoriasis tissue, the overlap did not increase when analyzing genes upregulated after repeated stimulation. These results suggest that chronic IgE-mediated exposure of mast cells may contribute to changes in tissue observed in chronic atopic conditions.

DISCUSSION

In this study, we aimed to characterize mast cell-intrinsic changes after repeated exposure to antigen, such as occurring in chronic allergy. We have used cultured human cord blood-derived mast cells which remain viable and stable in phenotype over several weeks, and showed that several changes in mast cells occur after repeated stimulation with anti-IgE. Obviously, in vitro stimulation is unlikely to capture all the changes that occur in vivo after repeated antigen exposure, as these are the result of interactions between a variety of cell types difficult to mimic in vitro. However, we hypothesized that in addition to those changes, cell-intrinsic changes as a consequence of repeated activation are likely to occur, and we were able to capture these changes using RNA sequencing.

To our knowledge, this is the first study to evaluate the effect of repeated FcεRI stimulation on human mast cells. Importantly, a high proportion of upregulated genes after repeated FcεRI triggering were related to changes in RNA expression in tissue of chronic allergic disorders, in particular chronic rhinosinusitis with nasal polyps. Our most highly upregulated gene CCL18 is highly linked to allergy, and was found to be expressed by mast cells in nasal polyps (*29*). Whereas some studies were able to show increased CCL18 mRNA expression by mast cells after a single anti-IgE stimulation, most studies failed to show secretion of CCL18 protein in response to acute anti-IgE stimulation (*30-32*). Our data indicate that robust expression and secretion of CCL18 in response to anti-IgE requires repeated or prolonged activation, and may explain how

expression of CCL18 in mast cells is induced in chronic allergic tissue. Besides recruitment of Th2 cells through upregulation of chemokines, several genes involved in T cell activation or antigen presentation to T cells were also upregulated in mast cells after repeated FcεRI triggering. Although the function of mast cells as antigen presenting cells is controversial (*33*), we and others have previously shown that human mast cells can function as antigen presenting cells through surface expression of HLA class II, and that tissue mast cells express HLA class II and costimulatory molecules (*34, 35*). Therefore, the upregulation of HLA class II observed after repeated FcεRI triggering suggests that repeated exposure to antigen may enhance this function of mast cells, thereby further contributing to T cell activation in chronic allergic tissue.

The GO-term "response to bacteria" was also highly upregulated after repeated stimulation of mast cells. Several studies have shown that combined stimulation of FcεRI and TLR can induce a synergistic cytokine response in mast cells and basophils, suggesting that this pathway could contribute to allergic exacerbations in the presence of pathogens (*36-39*). We now show that the response of mast cells to TLR-4 triggering is also enhanced after repeated stimulation through FcεRI, indicating that antigen exposure of mast cells can lead to long-term changes in mast cells associated with increased responsiveness to bacteria or LPS, thereby potentially contributing to allergic exacerbations.

These longterm changes in response mode of mast cells are likely regulated by transcription factors. Significant enrichment for target genes of CEBPA, RXRA, BATF were observed, suggesting that these may contribute to the modified RNA expression profile after repeated FcεRI stimulation. CEBPA is a well-known transcription factor involved in myeloid differentiation and is known to antagonize mast cell fate at the progenitor stage (*40, 41*). Likewise, retinoic acid, which can act through RXRA in mast cells, was shown to reduce mast cell differentiation during the progenitor stage, but did not affect mast cell lineage stability after full maturation (*42*). The function of these transcription factors has not been studied widely in mature mast cells. Interestingly, one study showed that overexpression of CEBPA in murine mast cells increased their response to bacterial stimulation (*43*), effects that we observed in mast cells after repeated FcεRI triggering as well. Our results therefore warrant further investigation of the transcriptional regulation of the function of mature mast cells in the context of chronic inflammation.

Whereas several immune-related pathways were upregulated, we observed an overall dampening of the FcεRI-triggered acute mast cell response after repeated triggering through FcεRI. Although mast cell degranulation was not affected, the expression of

many genes was reduced. Similar observations have also been made in the macrophage response to repeated LPS stimulation (*44*), where the gene-specific effects have been named tolerized genes (for genes that display a reduced expression) or non-tolerizable (for genes that display an unaltered expression).

Although we have not studied the mechanism of the dampened response observed in mast cells repeatedly exposed to FcεRI-triggering, it is interesting that several inhibitory receptors which can dampen FcεRI-induced mast cell activation were upregulated, including LAIR1, LILRB2, LILRB3, and VSTM1, providing a possible explanation for the dampened mast cell responses observed (*45, 46*). Interestingly, several inhibitory receptors are also upregulated in tissue from allergic patients (Online Repository Table E16), suggesting that dampening of inflammatory responses through upregulation of inhibitory receptors might represent an attempt to maintain tissue homeostasis (*47*).

In conclusion, our study provides a thorough analysis of mast cell-intrinsic changes after repeated FcεRI triggering. These changes were associated with an increase in release of chemotactic factors, including CCL18, increased antigen presentation to T cells, and increased response to bacterium, showing that the mast cell response modes are modulated after repeated antigen exposure. Importantly, these changes underlie several pathogenic mechanisms in chronic allergy, and were associated with changes in tissue of chronic allergic conditions. Therefore, our study may provide insight into the mechanisms of mast cell involvement in chronic allergic responses.

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SUPPLEMENTARY METHODS

CORD BLOOD-DERIVED MAST CELLS

Heparinized cord blood was obtained through the department of Obstetrics of the Leiden University Medical Center (Leiden, The Netherlands), and was performed in accordance with the Declaration of Helsinki. Mononuclear cells were isolated using a standard Ficoll procedure, after which $CD34^+$ hematopoietic stem cells were isolated with CD34 microbeads (Miltenyi Biotec). Isolated $CD34^+$ stem cells were differentiated into mast cells using serum-free medium (StemPro 34 + supplement, Gibco) with 5 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50,000 cells/mL as described (48, 49). Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. In addition, 50 ng/mL SCF was added once weekly. When cell number exceeded $1x10^6$/mL, additional medium was added instead of replacing the medium.

All recombinant cytokines were obtained from Peprotech. After six weeks, mast cells were incubated with the same medium containing 10% FCS for one week to induce maturation, and were maintained in this medium throughout the experiment. The purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI, CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99% (Supplementary Fig 1A-D). Morphology of mast cells was analysed using cytospins stained with Toluidine blue.

RNA ISOLATION

RNA of four samples (donor 1) was isolated from pelleted Mast Cells cultures with a standard Trizol protocol, modified for samples with high proteoglycan content (according to manufacturers' instructions, Life Technologies). However, increased RNA yields were observed if RNA was isolated with the mirVana miRNA Isolation Kit (Ambion). As a result, RNA from all other donors were isolated with mirVana isolation kit.

cDNA SYNTHESIS AND QPCR

150 to 500ng RNA was used for cDNA synthesis with SuperScript® III Reverse Transcriptase (Life Technologies) according to manufacturer's standard instructions in a total volume of 20µl. cDNA samples were diluted 1:18 in distilled water.

The primer sequences, primer concentrations, and Melting Temperatures used for qPCR are shown in Online Repository Table E1. All qPCR reactions were performed with 4µl SensiFAST™ SYBR® No-ROX (Bioline), and 3µl diluted cDNA. qPCRs were performed in a Biorad CFX-384 with the following protocol: 2 min 95,0°C; 45 cycles of 5s 95,0°C, 10s Tm, 25s 72°C; followed by 10s 95,0°C; 7: Melt Curve 65,0°C to 95,0°C: Increment 0,5°C/5s. Relative expression was calculated with ddCq method normalized to housekeeping gene RPL5. Similar results were obtained when normalization was done using B2M as housekeeping gene.

RNA-SEQ

Four RNA samples from donor 1 were subjected for RNA sequencing with Illumina HiSeq 2000 at local facility LGTC (paired end, strand specific, 2x100bp). For the second and third donor, sixteen samples were sequenced with Illumina HiSeq 2000 at BGI, China (2x 90bp). Sequence libraries were prepared based on Illumina TruSeq protocol modified by the sequence center.

In brief, RNA samples were DNAse treated and mRNA was selected with oligo-dT beads. mRNA was fragmented by heat incubation. Standard 1st strand synthesis was performed with Superscript III (ThermoFisher, Life Technologies) and T nucleotides and samples were subjected to RNAseH treatment. Second strand synthesis with U nucleotides was performed and dsDNA fragments were End repaired, 3'phosphorylated and dA-tailed. Adapters were ligated and second strand was removed by USER digest. Libraries were pre-amplified and sequenced on Illumina Hiseq.

RNA-SEQ QUANTIFICATION

Analysis of RNASeq data was done using the in-house pipeline Gentrap (version 0.4.3; https://git.lumc.nl/rig-framework/gentrap). In particular, adapters were detected with FastQC (version 0.10.1) and removed by cutadapt (version 1.1) if the overlap is greater than 12 base pairs. Reads were trimmed with sickle (version 1.2) using the quality threshold of 33 and minimum length after trimming greater than or equal to 50. Only cleaned and trimmed reads were used for downstream analysis. GSNAP (version 2014-5-15) was used to align high quality reads to the human reference genome (version

GCA_000001405.15_GRCh38, without alternative loci) (50). Alignment quality metrics were collected using Picard (http://picard.sourceforge.net version 1.86).

A minimum of 18 million reads per sample were obtained, and at least 15 million reads remained after quality control (Online Repository Table E2). Greater than 94% of cleaned reads were mapped to the GRC38reference genome. Exon annotation was based on the RefSeq transcript annotation downloaded from UCSC genome browser. As expected for polyA$^+$ RNA, >70% of mapped reads aligned to annotated exons.

Sample to sample spearman correlation coefficient (r) ranged from 0.90 to 0.96 between independent experiments and from 0.97-0.99 between technical replicates (Supplementary Fig 2 A). We observed a good correlation of gene expression in unstimulated samples with the expression of genes in skin MC expanded in vitro or analysed directly ex vivo as present in public databases(22) (R^2 = 0.76 and 0.70 respectively; Supplementary Fig 2 B). Furthermore, genes differentially expressed in anti-IgE stimulated mast cells as compared to naïve mast cells largely overlapped with those found in the CAGE study with skin-derived mast cells and those previously reported to be expressed by peripheral blood-derived mast cells (Supplementary Fig 2 C) (51).

Gene expression (number of reads per gene) was counted by htseq-count (HTSeq version 0.6.1, stranded = no, mode = intersection-nonempty, minimum alignment quality = 10) using RefSeq annotation including non-protein coding regions (accessed at 2014-09-25) (52). Due to overlapping annotations in RefSeq, genes with no unique features were excluded. As a result, 25,109 genes were used for RPKM (Reads per Kilobase per Million) calculations using EdgeR.

DIFFERENTIAL EXPRESSION

Genes with RPKM >3 in at least one sample for each donor (9,955 genes) were used in differential expression analysis to reduce noise from genes with very low expression levels. To identify pairwise differentially expressed genes, we used the generalized linear model (GLM) implemented in the EdgeR package (version 3.4.2) (21). In particular, both the treatment conditions and donor pairing were included in the GLM design to account for all systematic sources of variation. The Cox-Reid profile-adjusted likelihood (CR) method was used to estimate gene-wise dispersion based on the give GLM design. After fitting to the GLM design, the GLM likelihood ratio test was performed to detect differentially expressed genes. The P-value for multiple testing was adjusted using the Benjamini-Hochberg correction with a false discovery rate (FDR) of 0.05.

GO TERM ENRICHMENT AND VISUALIZATION

To understand the biological functions of gene sets, we performed GO term enrichment analysis using DAVID.(23, 53) Enriched GO terms were then imported into the Cytoscape (version 3.10) plugin EnrichmentMap (version 2.0) (24, 25). Terms with FDR < 0.01 were visualized. The threshold for overlapping terms was set at 0.6-0.7.

TRANSCRIPTION FACTOR ANALYSIS

From the list of differentially expressed genes, transcription factors were identified by the GO term "transcription factor activity" using DAVID. In order to identify whether target genes of these transcription factors were significantly enriched in the differentially expressed genes after repeated FcεRI triggering, we used the analysis tools Cscan and Pscan, identifying binding sites of transcription factors (Cscan) or predicted binding affinity (Pscan) to promoter regions of the gene lists from mast cells (26, 27, 54, 55). Target genes of transcription factors for which binding has been shown in Chip-Seq experiments were generated using Cscan.

MEASUREMENTS OF PROTEIN EXPRESSION

For flow cytometry, cells were incubated with fluorochrome-conjugated antibodies for CD117, IgE, FcεRI, CD63, CD203c, and HLA-DR diluted in PBS 0,5% BSA at 4 ºC for 30 min. Intracellular staining for tryptase and chymase was performed as described (49). Flow cytometric acquisition was performed on FACS Calibur (BD) and Fortessa (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

Cytokines in culture supernatants were measured using Milliplex assays (Millipore) and ELISA for IL-8 (eBioscience), and CCL18 (R&D). Lipid mediators leukotriene C_4 (LTC_4) and Prostaglandin D_2 (PGD_2) were analysed by competitive ELISA (Neogen and Cayman Chemical respectively).

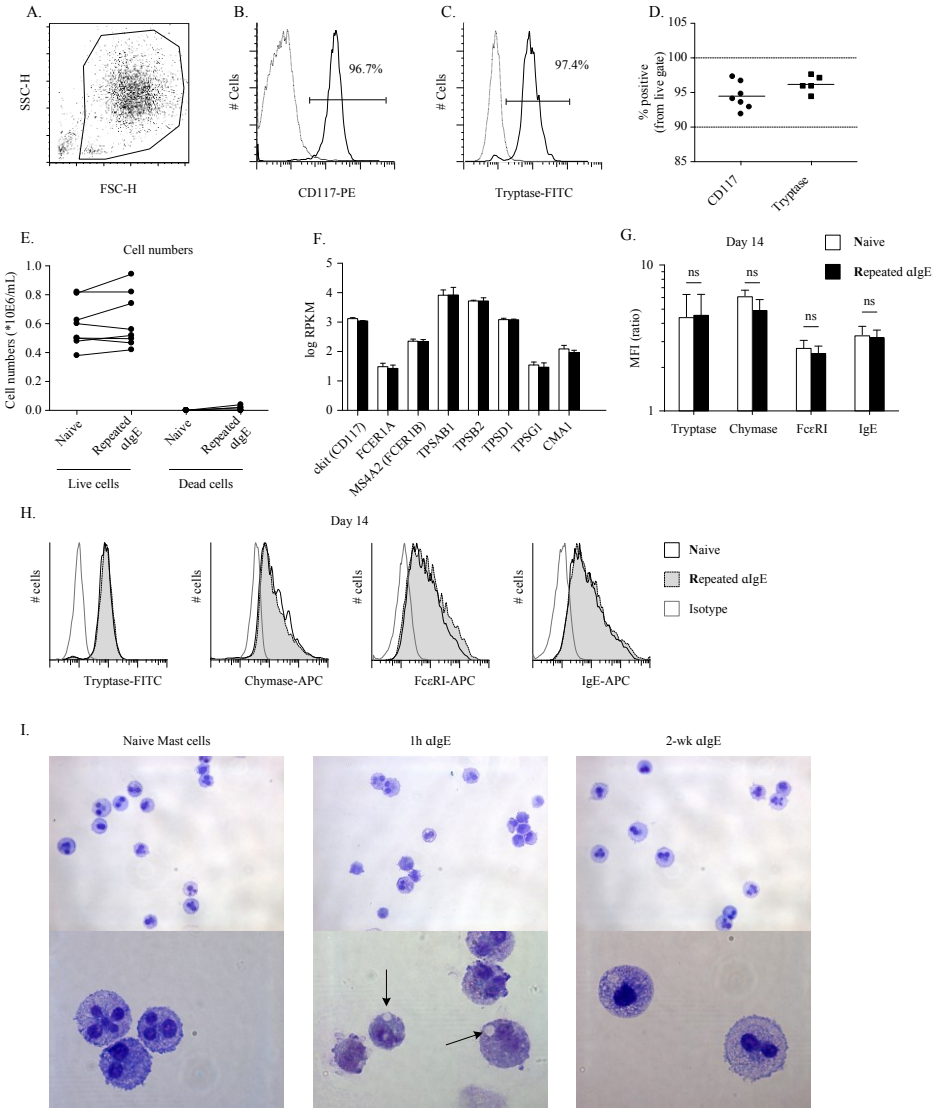
PUBLICLY AVAILABLE GENE EXPRESSION DATA

For comparison of gene expression in mast cells to other studies, public available RNA sequencing data was obtained from the FANTOM5 project deep cap analysis gene expression (CAGE) sequencing of skin-derived mast cells (22). Microarray data was obtained from a study of peripheral blood-derived mast cells (51).

For comparison to gene expression in atopic tissues, we searched Pubmed for microarray or RNA sequencing studies of tissue from patients with any chronic atopic or allergic disease (Online Repository Table E3) (56-62). Only those studies which examined whole tissue (as opposed to e,g, epithelial cells) were selected. Furthermore, the selection criteria

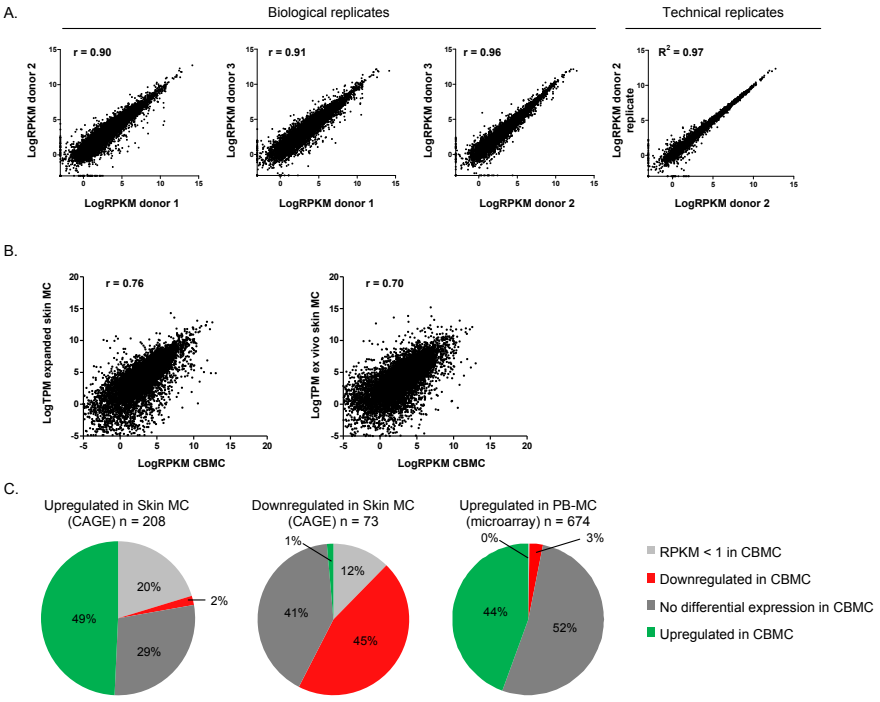
included the requirement for studies that had included patients with confirmed atopy or allergy. Expression data from psoriasis patients was used as control (63). A list of inhibitory receptors was based on known expression by mast cells and additional genes derived from the literature (Online Repository Table E16) (64).

SUPPLEMENTARY FIGURES



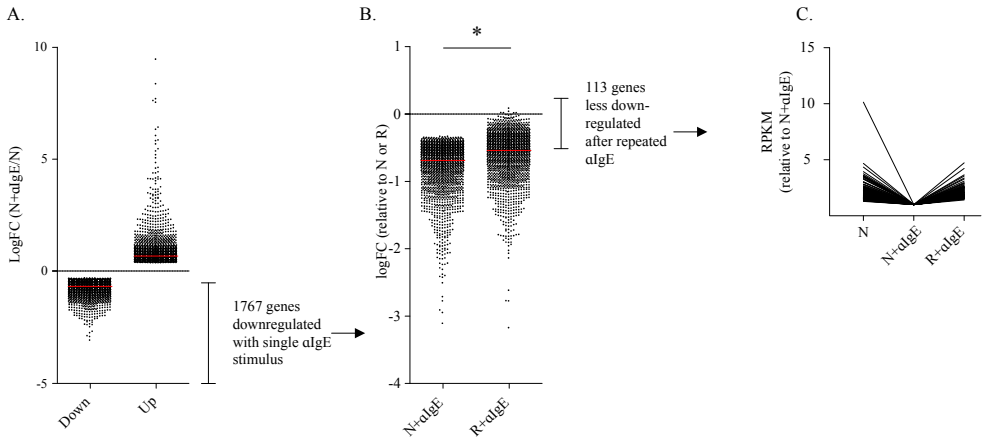
Supplementary Figure 1. Mast cell characteristics during culture. A-D) Gating strategy and purity of mast cells based on flow cytometry analysis of CD117 and tryptase.

A) Gating of live cells (excluding debris) based on forward scatter (FSC) and side scatter (SSC). B,C) Expression of CD117 and intracellular tryptase in live cells, gated as in A. Dotted line indicates isotype control. D) Summary of the percentage of CD117 and tryptase positive cells within the live gate in n=7 (CD117) or n=5 (tryptase) mast cell donors. E) Cell numbers after two-week culture of cord blood derived mast cells. Mast cells were cultured for two weeks with control medium (Naive) or anti-IgE (Repeated αIgE). The starting cell number was 1*10E6/mL medium. Numbers of live and dead cells at the end of two weeks stimulation were counted using Trypan Blue. Ns = no significant differences observed when comparing Naive mast cells versus anti-IgE-treated mast cells, analysed using paired samples T-test. N = 8 independent experiments with different mast cell donors. F) Transcripts for mast cell maturation markers after two weeks of FcεRI stimulation. Mast cells were cultured as described in E. Gene expression was obtained using RNA from 3 independent experiments performed with different mast cell donors. Results are shown as mean +/- SEM. RPKM = Reads Per Kilobase per Million. G,H) Intracellular tryptase and chymase expression and cell surface FcεRI expression and IgE binding at the end of the two weeks in naive mast cells (N) or mast cells stimulated for two weeks with anti-IgE (R). Representative flow cytometry plots (H) and a summary (G) are shown. MFI ratio is calculated as a ratio of the median fluorescence intensity (MFI) of staining divided by the MFI of the isotype control for each individual sample.I) Cytospins of mast cells were stained with Toluidine blue. Representative images of naive mast cells, mast cells stimulated for 1h with anti-IgE, and mast cells stimulated for 2 weeks with anti-IgE, Magnification: 20x (top) and 100x (bottom). Arrows indicate sites of degranulation.

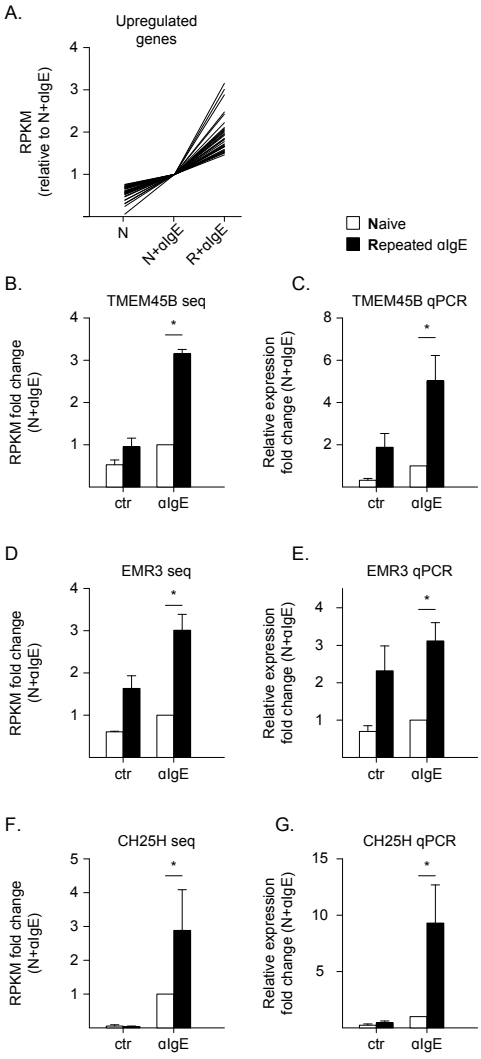


Supplementary Figure 2. Comparison of RNA sequencing results in our study with public data on mast cell transcriptome.

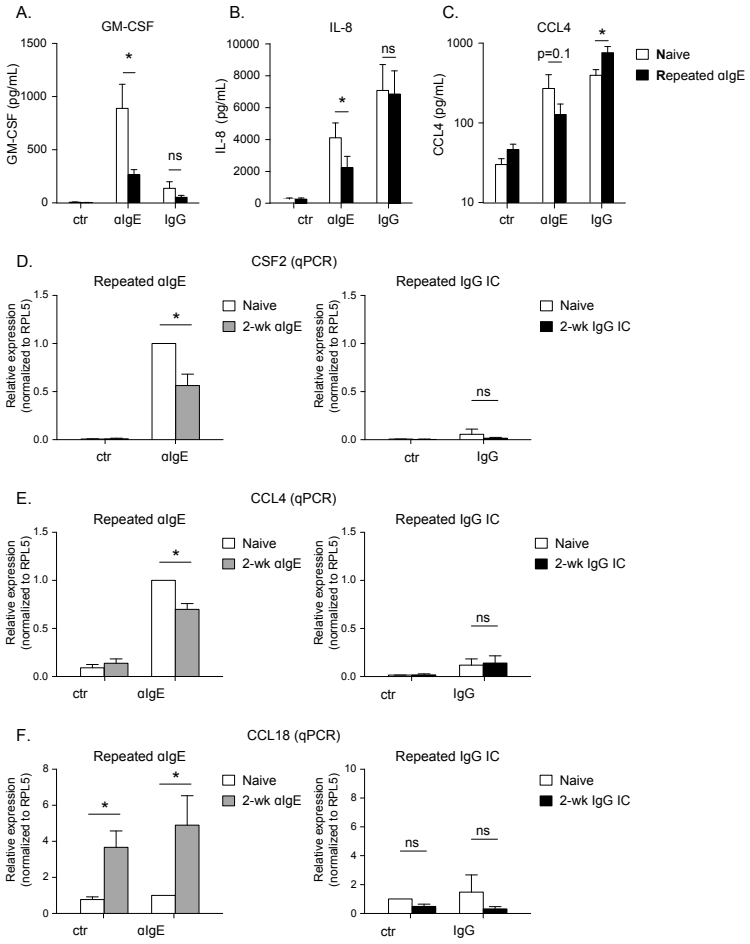
A) Spearman correlation of expression values (logRPKM) in biological or technical replicates. Each dot indicates an individual gene. B) Spearman correlation of expression values obtained in our study (CBMC) compared to skinMC expanded in vitro or analysed directly ex vivo, obtained through publicly available data from CAGE sequencing. Each dot indicates an individual gene. C) Overlap of differentially expressed genes upon single anti-IgE stimulation in our study, with publicly available data from CAGE sequencing (left, middle), or microarray (right). Upregulated genes in skin MC (left), downregulated genes in skin MC (middle), or upregulated genes in PB-MC (right). The number indicated above the piechart is the number of genes differentially expressed in those studies. CAGE: cap analysis gene expression; CBMC: cord blood-derived mast cells; MC: mast cells; PB-MC: peripheral blood-derived mast cells; RPKM: reads per kilobase per million; TPM: transcripts per million.



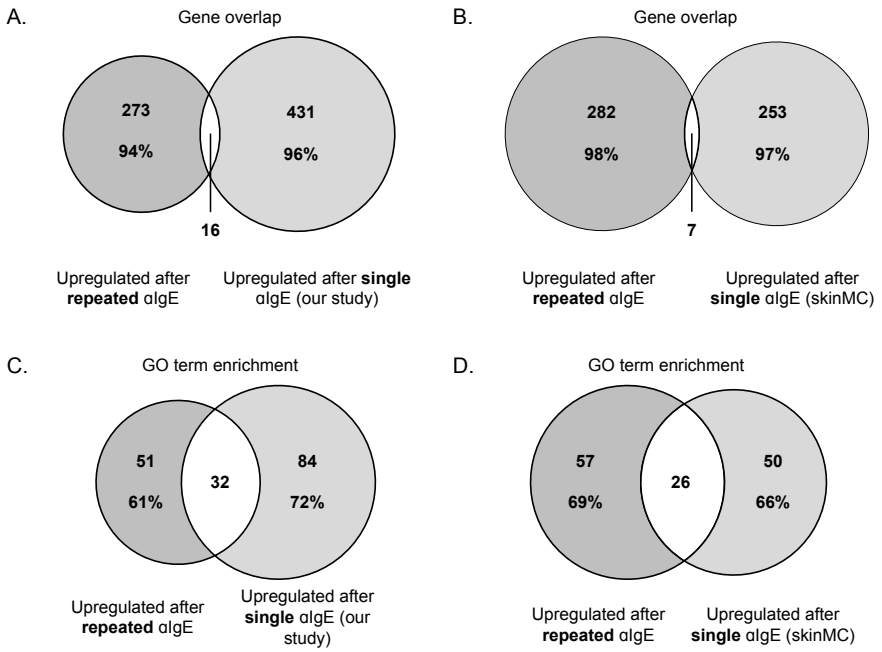
Supplementary Figure 3. Dampened downregulation after repeated stimulation. A) Gene expression obtained using RNA sequencing in response to a single 6 hour anti-IgE stimulation (N+αIgE), compared to control mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. B) Genes which were significantly downregulated after a single anti-IgE were selected as shown in A), where each dot indicates a different gene. 113 genes had significantly less downregulation after repeated stimulation. C) Genes which had significantly dampened downregulation after repeated anti-IgE, compared to single anti-IgE using differential expression analysis. Each line represents a single gene, and data is shown as relative RPKM normalized to single anti-IgE stimulation (N+αIgE). Asterisks indicate significant differences obtained using paired T-test $p<0.05$ (B).N = naive mast cells; N+αIgE = a single 6 hours stimulation of naïve mast cells with anti-IgE; R = repeated anti-IgE stimulation for two weeks; R+αIgE = 6 hours stimulation with anti-IgE after repeated anti-IgE stimulation.



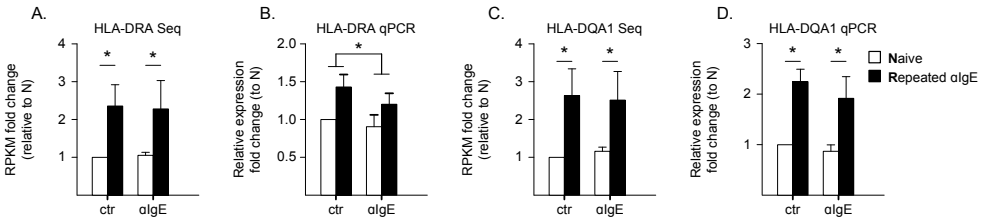
Supplementary Figure 4. Upregulation of FcεRI-responsive genes after repeated anti-IgE. A) Genes which are significantly upregulated after repeated anti-IgE, compared to single anti-IgE using differential expression analysis. Each line represents a single gene, and data is shown as relative RPKM normalized to single anti-IgE stimulation (N+αIgE). B, D, and F) Gene expression of TMEM45B, EMR3, and CH25H, obtained using RNA sequencing. Data is shown as RPKM fold change relative to single anti-IgE stimulation (N+αIgE). C, E, and G) Gene expression of TMEM45B, EMR3, and CH25H, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to single anti-IgE stimulation (N+αIgE). Data are represented as mean ± SEM from n=3 (B, D, and F) or n=5 (C, E, and G) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (B, D, and F) or repeated measures ANOVA with Bonferroni posthoc test p<0.05 (C, E, and G). N = naive mast cells; N+αIgE = a single 6 hours stimulation of naïve mast cells with anti-IgE; R = repeated anti-IgE stimulation for two weeks; R+αIgE = 6 hours stimulation with anti-IgE after repeated anti-IgE stimulation.



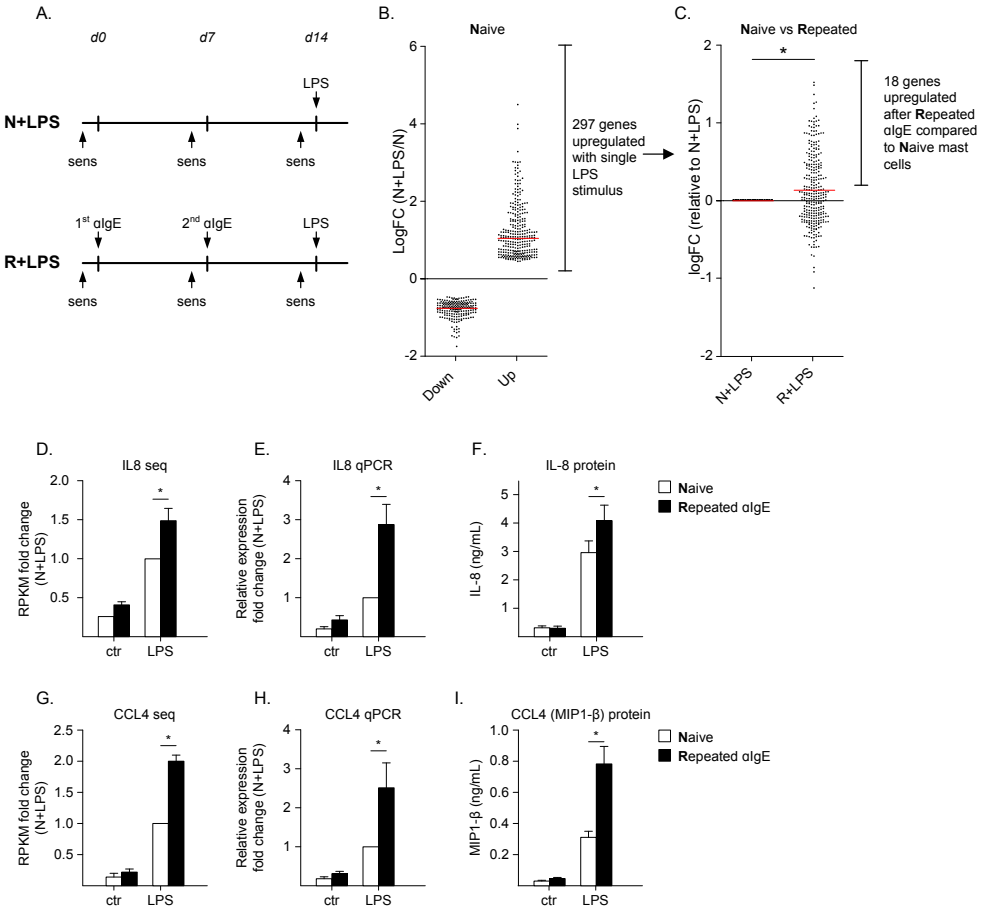
Supplementary Figure 5. Difference between stimulation with anti-IgE and IgG immune complexes. A-C) Mast cells were sensitized and stimulated with anti-IgE as described in the manuscript. Mast cells were re-stimulated at day 14 with 1 µg/mL anti-IgE or platebound IgG immune complexes. After 24 hours, mast cells supernatant was harvested, and cytokines were measured using ELISA (IL-8) or multiplex assays. Mean +/- SEM of pooled data from 3 independent experiments with different mast cell donors, each performed in duplicate. Asterisks indicate significant ($p<0.05$) differences between naïve mast cells and those treated for two weeks with anti-IgE, analysed using paired samples T-test. Ns = not significant. D-F) Mast cells were stimulated for 2 weeks with anti-IgE (left) or with IgG immune complexes (right). At day 14, mast cells were re-stimulated for 6 hours with anti-IgE or IgG immune complexes respectively, after which RNA expression was analysed by qPCR as described in the manuscript. Mean +/- SEM of pooled data from 2 independent experiments with different mast cell donors each performed in duplicate. Data is shown as relative expression normalized to household keeping gene RPL5 as a fold change to a single aIgE stimulation (N+aIgE). Asterisks indicate significant ($p<0.05$) differences between naïve mast cells and those treated for two weeks with anti-IgE, analysed using Two-way ANOVA. No significant differences (ns) were observed in expression of these genes after two-week stimulation with IgG immune complexes.



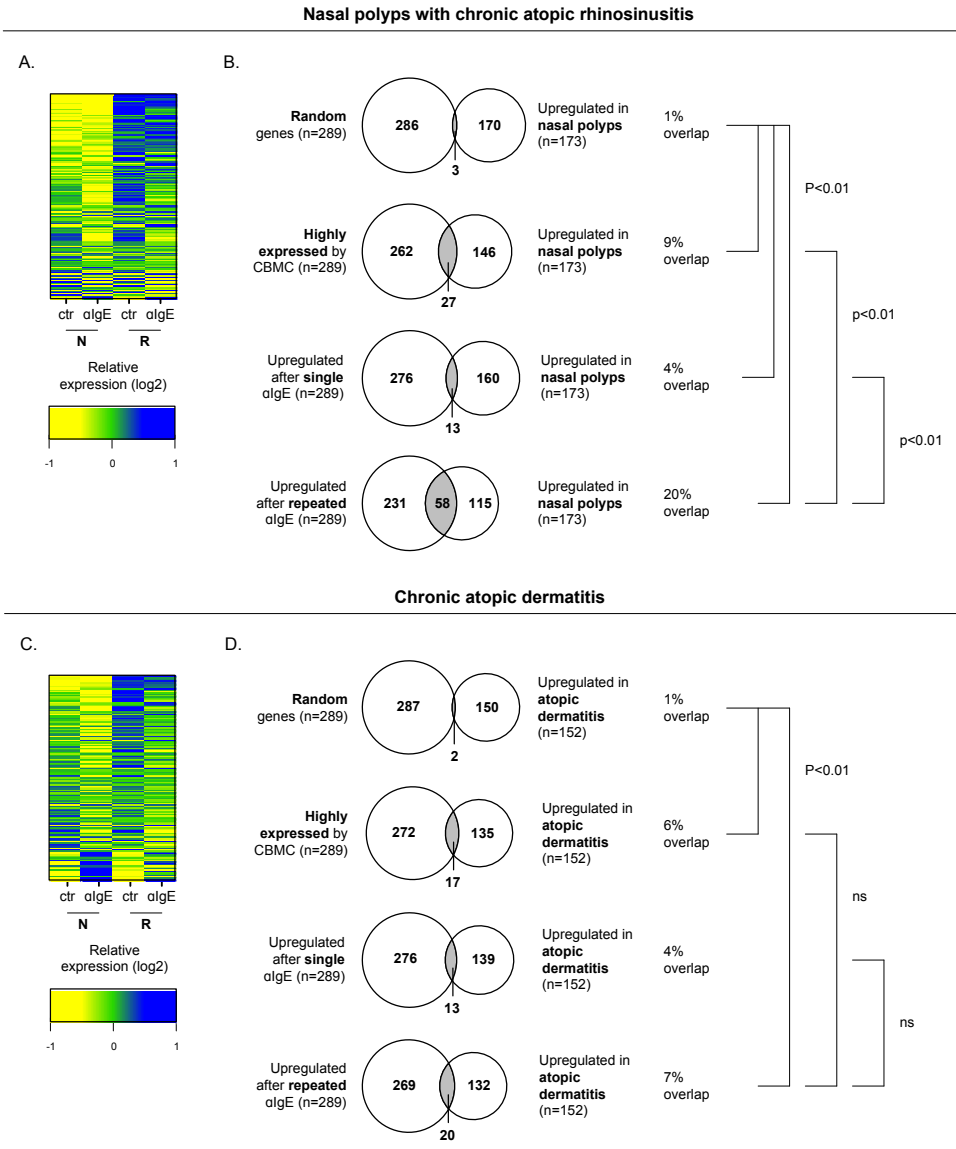
Supplementary Figure 6. Overlap in genes and enriched pathways between repeated and single stimulation of mast cells. A-B) Overlap of genes upregulated after repeated anti-IgE and genes upregulated after a single anti-IgE stimulation identified using differential expression analysis in this study (A) or in skin-derived mast cells from CAGE sequencing (B). C-D) Overlap of enriched GO terms after repeated anti-IgE and genes upregulated after a single anti-IgE stimulation identified using differential expression analysis in this study (C) or in skin-derived mast cells from CAGE sequencing (D)



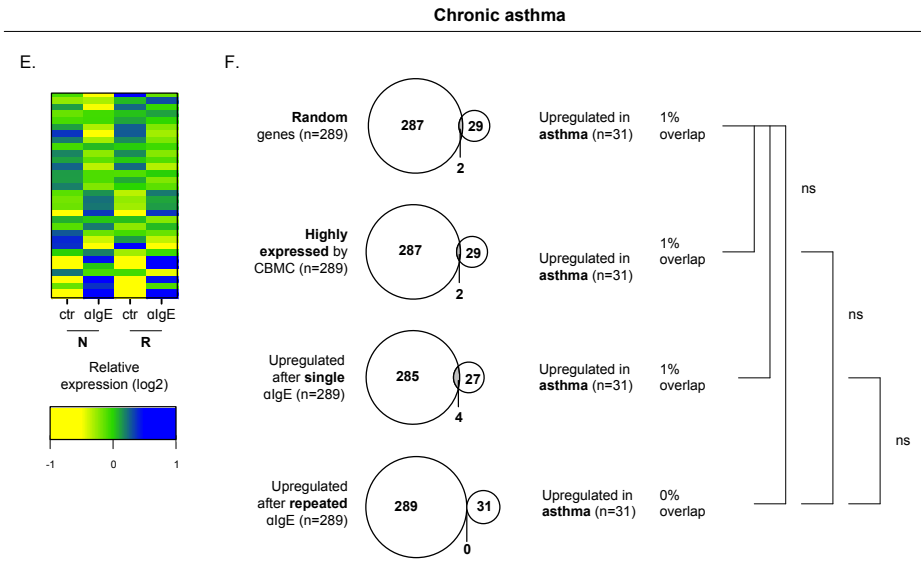
Supplementary Figure 7. HLA-class II RNA expression by qPCR. A, and C) Gene expression of HLA-DRA and HLA-DQA1, obtained using RNA sequencing. Data is shown as RPKM fold change relative to naïve mast cells (N). B, and D) Gene expression of HLA-DRA and HLA-DQA1, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change to naïve mast cells (N). Data are represented as mean ± SEM from n=3 (A and C) or n=5 (B and D) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (A and C), or repeated measures ANOVA (B) with Bonferroni posthoc test p<0.05 (D).



Supplementary Figure 8. Response to LPS is enhanced after repeated activation. A) Experimental setup of repeated stimulation of mast cells. Human cord blood-derived mast cells were stimulated for two weeks with 0.2 µg/mL anti-IgE (Repeated αIgE) or control medium (Naive). At the end of the two weeks, mast cells were stimulated with LPS (N+LPS and R+LPS). B) Gene expression obtained using RNA sequencing in response to a single 6 hour LPS stimulation (N+LPS), compared to Naive mast cells (N). Log fold change (LogFC) relative to Naive (N) mast cells of the differentially expressed genes is shown. C) Genes which were significantly upregulated after LPS stimulation were selected as shown in B), where each dot indicates a different gene. Data are shown as fold change relative to N+LPS. D, and G) Gene expression of IL8 and CCL4, obtained using RNA sequencing, 6 hours after stimulation with LPS. Data is shown as RPKM fold change relative to LPS stimulation in naive mast cells (N+LPS). E, and H) Gene expression of IL8 and CCL4, obtained using qPCR. Data is shown as relative expression normalized to housekeeping gene RPL5, as a fold change LPS stimulation in naïve mast cells (N+LPS). F, and I) Protein levels of IL-8 and CCL4 (MIP-1β) in supernatant, 24 hours after stimulation with LPS of naive mast cells or mast cells which were exposed to anti-IgE for two weeks. Data are represented as mean ± SEM from n=3 (D, and G) or n=5 (E, F, H, and I) independent experiments. Asterisks indicate significant differences obtained using differential expression analysis FDR<0.05 (D and G) or repeated measures ANOVA with Bonferroni posthoc test p<0.05 (E, F, H, and I).



Supplementary Figure 9. Association of upregulated genes with chronic allergic diseases. A, C, E) Genes upregulated in tissue of atopic rhinosinusitis with nasal polyps (A), atopic dermatitis (C) or atopic asthma (E) patients were selected from public databases. Relative expression of these genes in mast cells exposed to repeated anti-IgE stimulation is shown as heatmap. B, D, F) Overlap of genes upregulated in tissue of atopic rhinosinusitis with nasal polyps (B), atopic dermatitis (D) or atopic asthma (F) patients with random genes (top), genes highly expressed by mast cells (second row), genes upregulated in mast cells after a single anti-IgE stimulus (third row), or genes upregulated in mast cells after repeated anti-IgE (bottom).



Supplementary Figure 9 *(cont.)*. Overlap was calculated as percentage of the mast cell or random gene list. P values indicated were derived from Fisher's exact test after correcting for multiple testing ($p<0.01$). The overlap with random genes was analysed 5 times with separate lists of random genes, and the highest overlap obtained in these five tests was used for comparison in this figure.