TH17 EXPANSION BY HUMAN MAST CELLS IS DRIVEN BY INFLAMMASOME-INDEPENDENT IL-1β

Chapter 9

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ABSTRACT

Mast cells (MC) are most well-known for their role in innate immune responses. However, MC are increasingly recognized as important regulators of adaptive immune responses, especially in setting the outcome of T cell responses.

In this study we determined the effect of MC on cytokine production by naive and memory human T helper cells. $CD4^+$ T cells were cultured with MC supernatant or control medium, after which cytokine production by T cells was determined. Supernatant of activated MC specifically increased the number of IL-17 producing T cells. This enhancement of Th17 cells was not observed in the naive $CD4^+$ T cell population suggesting MC affected memory Th cells. The effect of MC was inhibited for approximately 80% by blocking antibodies to IL-1β and the recombinant IL-1 receptor antagonist anakinra. Importantly, secretion of active IL-1β by mast cells was independent of caspase activity indicating Th17 expansion by mast cells occurred through inflammasome-independent IL-1β.

Together, these studies reveal a role for human MC in setting the outcome of T cell responses through release of caspase-independent IL-1β, and provide evidence for a novel contribution of MC in boosting the Th17-axis in mucosal immune responses.

INTRODUCTION

Recent research has suggested an important role for mast cells in $CD4^+$ T cell responses. We and others have shown that human mast cells can function as antigen presenting cells, by taking up and processing antigens and subsequent activation of $CD4^+$ T cells via HLA class II molecules.(*1-3*) Although the precise contribution of mast cells in T-cell activation is still controversial, mast cells often colocalize with $CD4^+$ T cells, both in lymphoid organs as well as peripheral tissues.(*2, 4*) Therefore, it is conceivable that mast cells can interact with $CD4^+$ T cells and drive skewing of specific T helper cell subsets, through their secreted cytokines and other mediators.

Due to their role in allergy, mast cells are often associated with Th2 responses, but recent data suggest they may also contribute to Th17 responses, for example through an indirect effect on dendritic cells, or through an OX40-mediated crosstalk with regulatory T cells (Tregs) as shown in mice.(*5, 6*) IL-9 and IL-33, two cytokines which can modulate mast cell responses, have been related to Th17-mediated pathology in mice.(*7, 8*) Furthermore, several mast cell derived cytokines have been implicated in Th17 skewing, including IL-6, TGFβ, and IL-1β.(*9*)

Importantly, mast cells are predominantly present at mucosal surfaces which are important sites for Th17 activation and immunity. Although Th17 cell activation at mucosal barriers is important in driving protection against bacteria, aberrant Th17 responses can also contribute to pathogenic processes, such as neutrophilic infiltration or airway remodeling in asthma.(*10-12*)

Although studies performed in mice point to a role of mast cells in Th17 cell biology, little is known of the contribution of mast cells to Th17 responses in humans, also because the cytokines produced by MCs can differ considerably between mice and man.(*13, 14*) Understanding of the pathways that contribute to activation and maintenance of memory Th17 cells in humans is particularly important for inflammatory diseases, as Th17 memory cells are thought to be the driver of chronic inflammation.(*15*)

Therefore, we aimed to evaluate the influence of mast cells on Thelper cell responses. Here, we show that activated mast cells are capable of expanding Th17 cells. Studies investigating the mode of action revealed that this occurred through a synergistic release of IL-1β upon combined TLR/FcεRI receptor triggering. Secretion of active IL-1β by human mast cells upon stimulation was independent of caspase activation, suggesting a novel mechanism of inflammasome-independent IL-1β release by mast cells. As allergic responses in mucosal tissue are often associated with both Th17 responses and involvement of mast cells, these results provide a link between inflammasome-independent IL-1β production and pathogenic Th17 responses.

MATERIALS AND METHODS

MAST CELL CULTURE

Buffy coats from healthy volunteers were obtained from the blood bank (Sanquin, The Netherlands). PBMCs 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 30 ng/mL IL-3, 100 ng/mL IL-6 and 100 ng/mL Stem Cell Factor (SCF) at 50.000 cells/mL as described.(*16*) Half of the medium was replaced weekly with serum-free medium containing 100 ng/mL IL-6 and 100 ng/mL SCF. All recombinant cytokines were obtained from Peprotech. After 6-8 weeks, the purity of mast cells was determined by flow cytometric analysis of CD117 (c-kit), FcεRI and CD203c and intracellular tryptase. The purity of mast cells ranged from 90-99% (Supplementary Figure 1).

MAST CELL ACTIVATION

Mast cells were sensitized using 0,1 ug/mL hybridoma IgE (Diatec, clone HE-1) for a minimum of 18 hours. Mast cells were thoroughly washed to remove the soluble IgE, after which IgE was crosslinked using 10 ug/mL polyclonal goat anti-human IgE (Nordic). Activation of TLR was achieved using 1 ug/mL LPS (Invivogen). Activation of Fc-gamma receptors was achieved by using platebound IgG as described before.(*17*) Mast cells were activated in StemPro medium + supplement in the presence of 100 ng/mL SCF for 24 hours, after which supernatant was harvested.

For inhibition of caspase, 20 uM Z-VAD-FMK (Sigma) was added to mast cells, 15 minutes prior to their activation, and the drug remained present throughout the 24 hour stimulation. The levels of lactate dehydrogenease (LDH) was analysed in mast cell supernatant (BioAssay Systems) as a measure of cell death.

$CD4^+$ T CELL SKEWING

For isolation of $CD4^+$ T cells, naive (CD45RO-negative) or total $CD4^+$ T cells were isolated from PBMCs by negative magnetic bead isolation (Miltenyi Biotec). The purity of isolated naive $CD4^+$ T cells was determined as $CD14^-CD3^+CD4^+CD45RA^+CD45RO^-$ cells, and the purity of total $CD4^+$ T cells was determined as $CD14^-CD3^+CD4^+$. The purity of isolated naive or total $CD4^+$ T cells was above 95% in each experiment.

Naive or total $CD4^+$ T cells were activated using 5 µg/mL platebound anti-CD3 (eBioscience; clone: OKT3) and 1 µg/mL soluble anti-CD28 (Sanquin; clone: CLB-CD28/1, 15E8) in the presence or absence of mast cell supernatant which was diluted 1:1 in IMDM/10% FCS. After 5 days of incubation, T cells were harvested, washed, and restimulated using 50 ng/mL PMA (Sigma) and 500 ng/mL Ionomycin (Sigma).

Cells were restimulated for 5 hours in the presence of 10 µg/mL brefeldin A (Sigma) for intracellular cytokine staining. For analysis of proliferation, isolated $CD4^+$ T cells were labeled with Cell Trace Violet (Invitrogen), after which they were cultured as mentioned above.

For blocking experiments, mast cell supernatant was incubated with 20 µg/mL mIgG1 isotype control (clone P3.6.2.8.1, eBioscience), IL-1RA (anakinra; kindly provided by dr. M Schilham), anti-IL1α (Bio-Techne, Minneapolis, MN), anti-IL-1β (clone CRM56, eBioscience), anti-IL-6R (Tocilizumab), or anti-TNF-α (Etanercept), for 1 hour at 37 ºC before adding the supernatant to the T cells.

IL-1β SECRETION AND ACTIVITY

The amount of IL-1β in mast cell supernatant was quantified using ELISA (Biolegend). The amount of IL-1α in mast cell supernatant was quantified using Luminex assays (Millipore). The IL-1 activity was measured by incubating the EL4.NOB-1 cell-line with mast cell supernatant or control medium as described,(*18*) after which secretion of mouse IL-2 by this cell-line was measured using ELISA (eBioscience). The NOB-1 cell-line was kindly provided by prof. Leo Joosten, Radboud University, Nijmegen, The Netherlands.

FLOW CYTOMETRY

The following antibodies were obtained from BD Biosciences: CD3-AlexaFLuor700 (clone UCHT1), CD4-APC (clone SK3), IL-10-PE (clone JES3-9D7), IL-13-APC (clone JES10-6A2), IFNγ-FITC (clone 25723.11), TNF-α-AlexaFluor488 (clone MAb11), mIgG1-FITC (clone X40), rIgG1-PE (clone R3-R4), mIgG1-AlexaFluor488 (clone MOPC-21); from eBioscience: IL-17A-AlexaFluor647 and -eFluor660 (clone eBio64CAP17), mIgG1-eF660 (clone P3.6.2.8.1) , mIgG1-PE (clone P3.6.2.8.1); and from R&D systems: IL-22-PE (clone 142928).

For surface staining, cells were incubated with fluorochrome-conjugated antibodies diluted in PBS 0,5% BSA at 4 ºC for 30 min. For discrimination between live and dead cells, cells were incubated with Fixable Viability Dye eFluor506 (eBioscience), prior to fixation and permeabilization. For intracellular cytokine staining, T cells were permeabilized using CytoFix CytoPerm Kit (BD Biosciences). After washing, cells were incubated with antibodies against intracellular cytokines and incubated at 4 ºC for 30 min. After washing, cells were suspended in 1% paraformaldehyde until flow cytometric aquisition on a FACS Calibur (BD), LSR-II (BD), or Fortessa (BD). Analysis was performed using FACS Diva (BD) and FlowJo software.

Cell proliferation was analysed as described.(*19*) Briefly, the % divided cells was defined as the probability that a cell has divided at least once from the original population. The division index was defined as the average number of cell divisions that a cell in the original population has undergone.

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical analysis was performed using SPSS PASW 17.0 and GraphPad Prism 4. For differences between two groups, student's T tests were performed. ANOVA with Bonferroni posthoc tests were used to analyse differences between multiple groups. P values of <0.05 were considered statistically significant.

RESULTS

ACTIVATED HUMAN MAST CELLS INDUCE TH17 EXPANSION

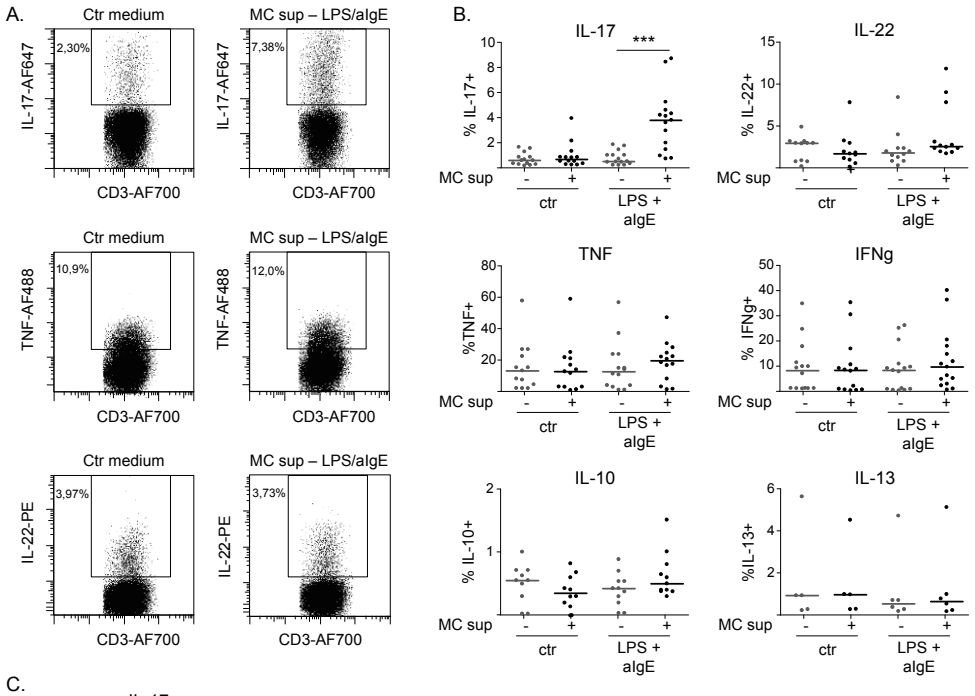
To evaluate the effect of mast cells on cytokine production by Th cells, isolated total $CD4^+$ T cells were incubated with supernatant obtained from mast cell cultures stimulated via TLR-4 and FcεRI. Medium containing the same concentrations of stimuli were used as control. After 5 days, cytokine production by T cells was analysed by intracellular flow cytometry upon restimulation with PMA/ionomycin (Figure 1A,B). Live $CD3^+$ T cells were gated as shown in Supplementary Figure 2. Of the analysed cytokines produced by responding T-cells, mast cells most significantly enhanced Th17 responses, characterized by a two- to eight-fold expansion of IL-17^+ cells (p=0.0001). Although enhancements of other Th cytokines like IL-22 and TNF-α were observed when T cells were activated in the presence of supernatant from some mast cell donors, these responses were not present in all T cell and mast cell donors and were therefore not statistically significant.

As enhancement of IL-17 responses could result from expansion of memory T cells or from *de novo* induction from naive Th cells, we next analysed the capacity of mast cell supernatant to skew IL-17 cytokine production from naive Th cells. Naive Th cells require specific cytokines for their differentiation into functional cytokine-producing Th cells. In contrast to the ability of mast cell supernatant to enhance Th17 responses from total Th cells, mast cells were not capable of inducing IL-17^+ T cells from naive Th cells (Figure 1C).

Together, these results indicate that activated mast cells can secrete substances that allow the increase of IL-17 production from T cell populations through an effect on memory Th cells. As only an increase in IL-17 producing T cells was noted, these results indicate a specific effect on IL-17 production but not on the production of other cytokines.

EXPANSION OF TH17 CELLS BY MAST CELLS IS INDEPENDENT OF PROLIFERATION

To address whether enhancement of Th17 responses by mast cells was driven by specific expansion of Th17 cells, we next analysed whether IL-17 producing T cells had undergone accelerated proliferation. To this end, cells were labeled with Cell Trace Violet fluorescent dye, and stimulated for 5 days with anti-CD3 and anti-CD28 in the presence of mast cell supernatant. At day 5, the proliferation history of IL-17, IFNγ, and TNF-producing cells was analysed. As shown in Figure 2, IL-17-producing cells had not undergone a more extensive proliferation compared to control, non-IL-17 producing T cells or IL-17-producing cells not exposed to supernatant of activated mast cells.



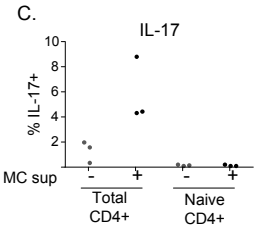


Figure 1. Supernatant of activated mast cells enhances Th17 responses. Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant, or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of BFA for 5 hours, to analyse their cytokine profile. A) Representative plots for intracellular cytokines IL-17, TNF-α, and IL-22. Live T cells were gated as shown in Supplementary Figure 2. B) Summary of 12 independent experiments, with each dot indicating a different mast cell supernatant. Asterisks indicate significant increase in the percentage of $IL\text{-}17^+$ T cells with supernatant from activated mast cells, as analysed using paired t-test (***p=0,0001). C) Summary of 3 independent experiments analyzing IL-17 production using intracellular cytokine staining of isolated total and naïve $CD4^+$ T cells, each treated with supernatant from a different mast cell donor.

Similar results were obtained when T cells producing other cytokines were analysed, although a small decrease in proliferation of IFNγ- and TNF-producing cells was noted in some donors (Figure 2C,D). Therefore, we conclude that the increase in Th17 cells by mast cells is independent of proliferation.

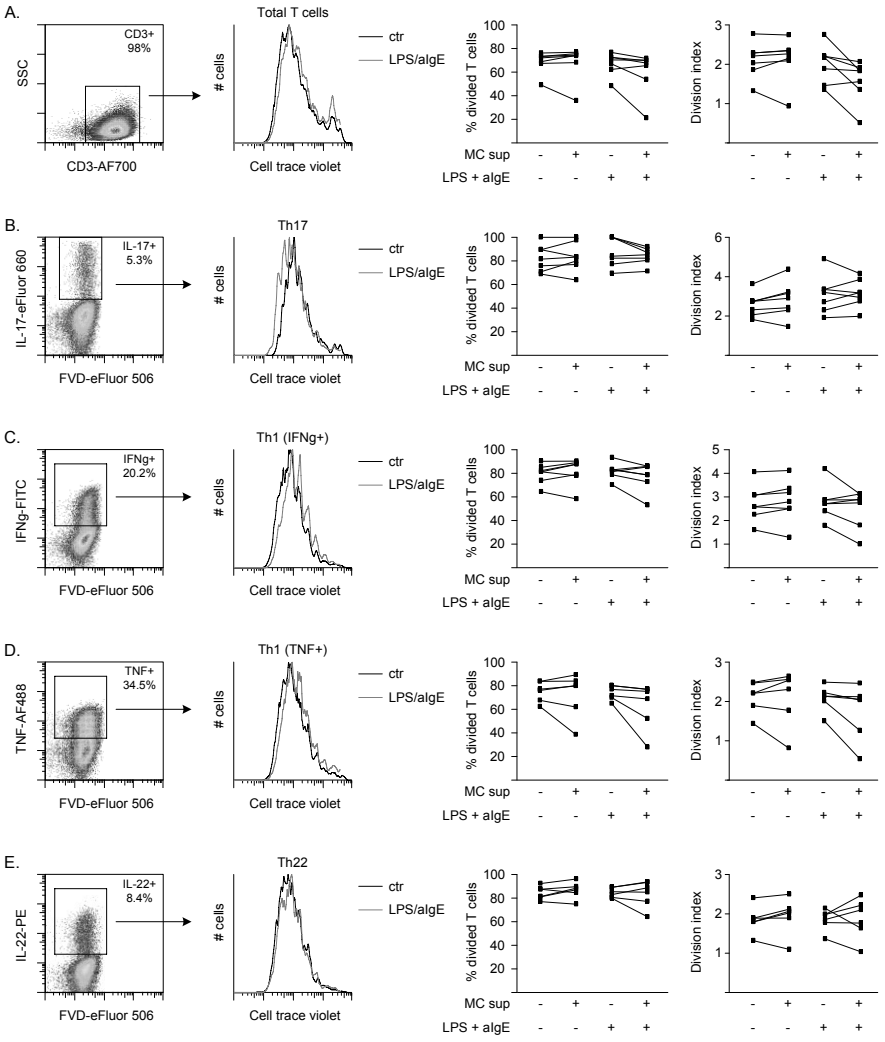


Figure 2: Proliferation of T helper cell subsets after culture with mast cell supernatant. Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were labeled with Cell Trace Violet and activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant, or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of brefeldin A for 5 hours, to allow the analysis of proliferation of different T helper cell subsets based on their cytokine profile. A-E) Representative flow cytometry plots of Cell Trace Violet as measure of proliferation (histograms) in T helper cell subsets based on cytokine staining, as shown in the plots on the left. Live T cells were gated as shown in Supplementary Figure 2. Summary of proliferation from 5 independent experiments is shown on the right, where each dot and line represents a different mast cell supernatant. The percentage of divided T cells is depicted as % of the input frequency, indicating how many cells from the original cell population have divided. Division index represents the average number of cell divisions that a cell in the original population has undergone. No significant differences were observed using paired samples t-test ($p<0,05$).

CHARACTERIZATION OF MAST CELL-INDUCED TH17 CELLS

Previous studies have identified different types of human Th17 cells with distinct effector function and differentiation requirements. Whereas memory Th17 cells have been shown to express IL-17 and TNF, other Th17 cell subsets co-express IFNγ, IL-22 or IL-10. Moreover, a substantial plasticity between these subsets has been reported, depending on the cytokine environment they are in.(*20-23*) To determine which of these subsets were preferentially expanded by mast cells, IL-17 staining of responding T cell cultures was combined with staining for TNF-α and IL-22 or IFNγ and IL-10 (Figure 3). Mast cell supernatants mainly enhanced the proportion of IL-17 single-positive cells, as well as the proportion of TNF-α/IL-17 double-positive T cells. Only a small minority of IL-17 producing cells produced IFNγ or IL-10.

Together, these results further confirm a specific effect on IL-17 production and suggest that mast cell-induced IL-17 producing T cells represent classical Th17 cells producing IL-17 and TNF, in the absence of other cytokines.

MAST CELLS PRODUCE ACTIVE IL-1β UPON TLR/FCεRI TRIGGERING

To better understand the mechanism responsible for strengthening of the Th17 axis by mast cells, we next wished to determine the role of cytokines secreted by mast cells in this process. Several cytokines (IL-1, IL-6, IL-23, TNF-α) have been described to enhance human memory Th17 responses. (*9*) As human mast cells are known to produce IL-1β, we first evaluated whether IL-1β could contribute to Th17 response enhancement, by using recombinant IL-1β (Fig 4A). As also noted by others, culturing T cells with recombinant IL-1β resulted in enhanced Th17 responses.(*21*) This effect could by blocked by the IL-1-receptor antagonist, anakinra, confirming the IL-1 receptor-dependency (Fig 4B).

We further wished to analyse whether mast cell supernatant contained IL-1β. As shown in Fig 4C, we observed increased production of IL-1β, but not IL-1α, in supernatant of activated mast cells, as detected by ELISA. To further confirm the presence of IL-1 in mast cell supernatant, the IL-1 activity was measured using the cell-line NOB-1, which produces mouse IL-2 in response to active IL-1. This cell-line responded to supernatant of activated mast cells, characterized by release of mouse IL-2 (Fig 4D). Importantly, treatment of the mast cell supernatant with inhibitors to IL-1α and IL-1β revealed a predominant inhibition of IL-1 activity by anti-IL-1β, suggesting that the majority of active IL-1 in mast cell supernatant is IL-1β (Fig 4D).

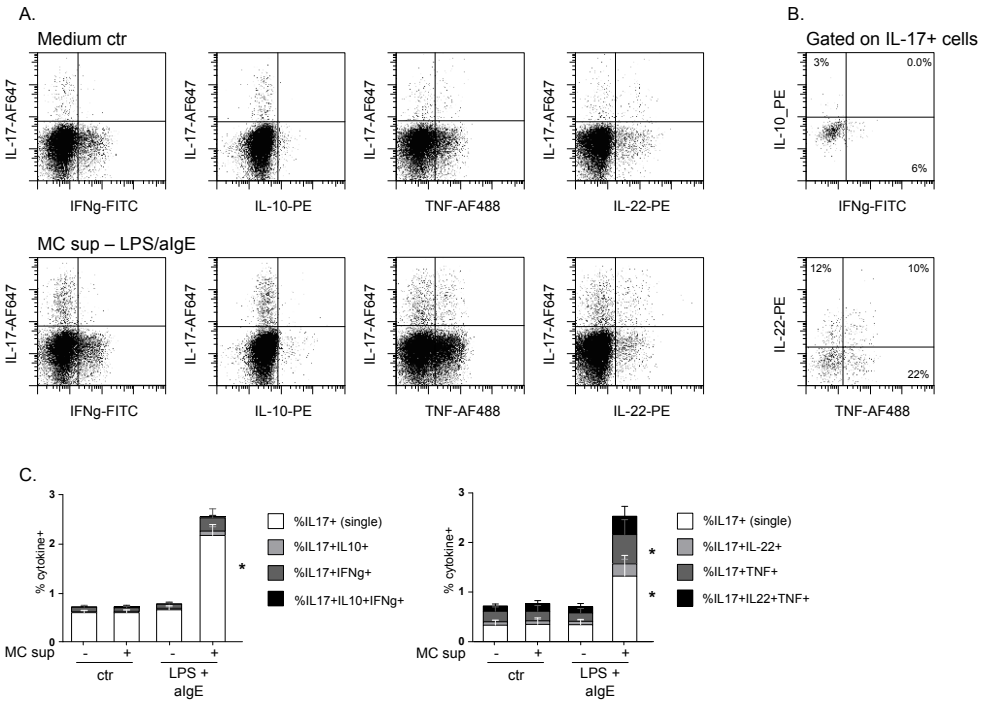


Figure 3. Characterization of Th17 cells induced by mast cells. Mast cells were left untreated, or were stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant, or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of brefeldin A for 5 hours, to analyse their cytokine production. A) Representative flow cytometry dot plots of combined staining for IL-17 with IFN-gamma, IL-10, TNF-α, IL-22. Live T cells were gated as shown in Supplementary Figure 2. B) Representative flow cytometry plots of IFN-gamma and IL-10, respectively TNF-α and IL-22 production by a T cell population, gated on IL-17^+ T cells, is shown. C) Summary of 7 independent experiments using mast cell supernatant from 9 different mast cell donors and T cells from 7 independent donors, showing the percentage of Th17 cell subsets defined by co-expression of other cytokines. Results are shown as mean +/- SEM. Asterisks indicate significant increase in the percentage of IL-17^+ T cell subset with supernatant from activated mast cells, analysed using two-way ANOVA and Bonferroni post hoc test (*p<0,001).

We next wished to analyse whether Th17 expansion was specific to FcεRI crosslinking or whether also other modes of mast cell activation induced IL-1 release and possibly a Th17-skewing phenotype. Therefore, we obtained supernatant of mast cells activated with IgG immune complexes (platebound) and LPS. We have previously shown that activation of mast cells through FcεRI or FcγRIIA in combination with LPS leads to a synergy in cytokine production.(*17, 24*)

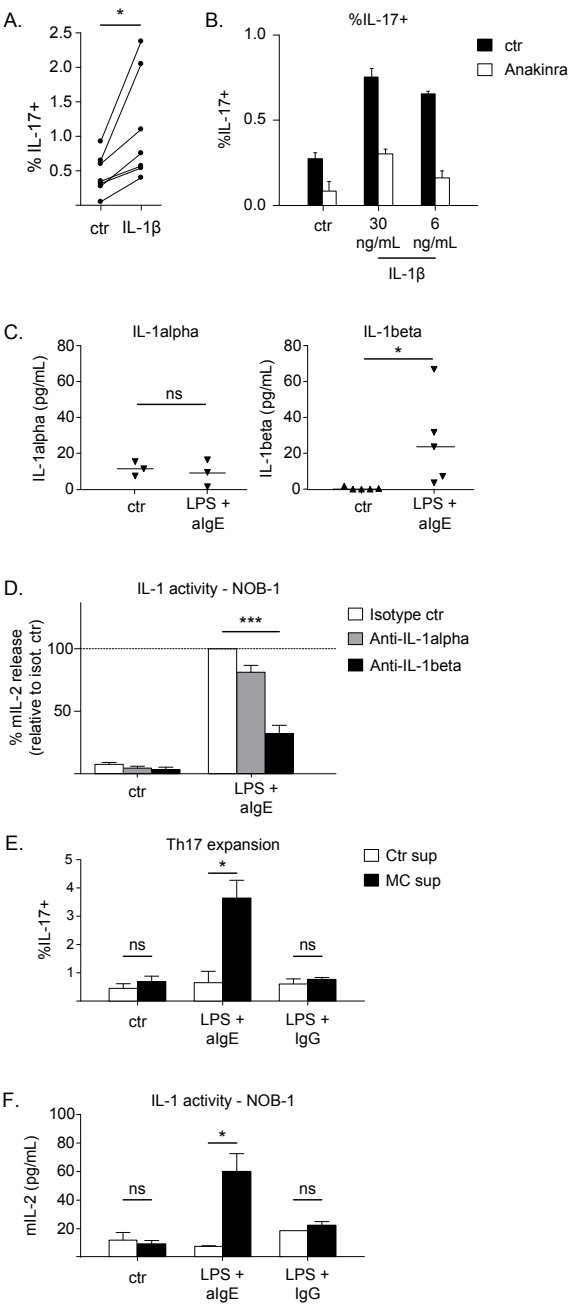


Figure 4. Mast cells produce active IL-1β upone TLR/FcεRI triggering. A,B) Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence or absence of recombinant IL-1β and with or without anakinra (IL-1 receptor antagonist). After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of BFA for 5 hours, to analyse their cytokine profile. A) Summary of 7 independent experiments. B) Representative example of blocking experiment using anakinra. Mean +/- SEM is shown. C) Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Levels of IL-1α and IL-1β in mast cell supernatant were measured using Luminex and ELISA, respectively. Summary of 3-5 different mast cell donors is shown. D) Mast cells were treated as in C). NOB-1 cells were cultured in the presence of mast cell supernatant, in combination of anti-IL-1α, anti-IL-1β or isotype control antibodies for 24 hours, after which the secretion of mouse IL-2 by NOB-1 cells was measured using ELISA. Percentage mIL-2 release that is shown was calculated relative to the amount of mouse IL-2 released by NOB-1 cells when treated with supernatant from mast cells activated with LPS + anti-IgE in presence of isotype control. Mean +/- SEM from 4 independent mast cell donors is shown. E) Mast cells were left untreated, stimulated with LPS and anti-IgE, or stimulated with LPS and platebound IgG immune complexes. T cells were cultured as in A) in the presence or absence of mast cell supernatant. Mean +/- SEM from 3 independent mast cell donors is shown. F) Mast cells were treated as described in E). NOB-1 cells were cultured in the presence of mast cell supernatant as described in D). Mean +/- SEM from 4 independent mast cell donors is shown. Asterisks indicates significant difference ($p<0.05$), using students T-test (A, C, E, and F) or Two-way ANOVA with Bonferroni posthoc test (D).

Interestingly, mast cell activation via FcγRIIA in combination with LPS did not induce increased Th17 responses as these were only observed when mast cells were activated with anti-IgE and LPS (Fig 4E). This correlated with IL-1 activity in mast cell supernatant, as this was only increased by stimulation through anti-IgE and LPS, and not by IgG immune complexes and LPS (Fig 4F). Together, these results indicate that mast cells secrete active IL-1β, specifically in response to anti-IgE in combination with LPS.

IL-1β RELEASE BY MAST CELLS IS INFLAMMASOME-INDEPENDENT

IL-1β is a cytokine that is initially produced as inactive pro-IL-1β, and therefore needs to be cleaved to generate the active molecule prior to secretion. The most well-known pathway leading to cleavage of IL-1β is the inflammasome-caspase-1 pathway. The specific pathways leading to IL-1β cleavage and secretion by mast cells are not known. Therefore, we analysed whether secretion of active IL-1β by mast cells was caspase-dependent, using a pan-caspase inhibitor (Z-VAD). The monocyte cell-line THP-1 was used as positive control. These cells secrete active IL-1β upon activation with LPS, due to inflammasome activation by endogenous ATP.(*25*) As depicted in Figure 5, secretion of active IL-1β by the monocyte cell-line THP-1 was almost completely reversed using Z-VAD (Fig 5A,B; left). In contrast, no inhibition of IL-1 activity was observed using this inhibitor in mast cells (Fig 5A,B; right).

Cell death can also lead to cleavage of pro-IL1β by mast cells, through aspecific release of pro-IL1β and chymase followed by extracellular cleavage. Therefore, LDH levels were quantified in mast cell supernatant as a measure of cell death. No significant increase in LDH levels were observed upon mast cell stimulation (Fig 5C), suggesting that the inflammasome-independent cleavage of IL-1β by mast cells is not due to cell death.

MAST CELLS INDUCE TH17 RESPONSES THROUGH INFLAMMASOME-INDEPENDENT IL-1β

To determine the contribution of IL-1β secreted by mast cells on Th17 responses, we next evaluated the effect of neutralizing IL-1β in mast cell supernatant, by using IL-1 receptor antagonist IL-1RA (anakinra) as well as a blocking antibody to IL-1β (Fig 6A-B). Both IL-1RA and anti-IL-1β almost completely reversed the expansion of the Th17 response induced by supernatant from activated mast cells. This effect was specific for Th17 responses, as no effect of these inhibitors on TNF production by T cells was observed (Fig 6B, right panel).

We next compared the blocking of IL-1β to that of potential other mast cell-derived cytokines described to enhance Th17 responses, IL-6 and TNF-α (Fig 6C). Whereas anti-IL-1β again led to a significant reduction in the number of Th17 cells, no effect was

observed with anti-IL-6R. Blocking of TNF-α led to an increase in the number of Th17 cells, indicating that TNF was not involved in the skewing towards Th17 responses by mast cells. As we observed that IL-1β release by mast cells was inflammasome-independent, we wished to confirm that Th17 responses induced by mast cell supernatant were not affected by caspase-inhibition. Indeed, Th17 expansion by mast cells was not affected by the pan-caspase inhibitor Z-VAD (Fig 6D).

Together, these results indicate that human mast cells can drive enhancement of Th17 responses through secretion of inflammasome-independent IL-1β.

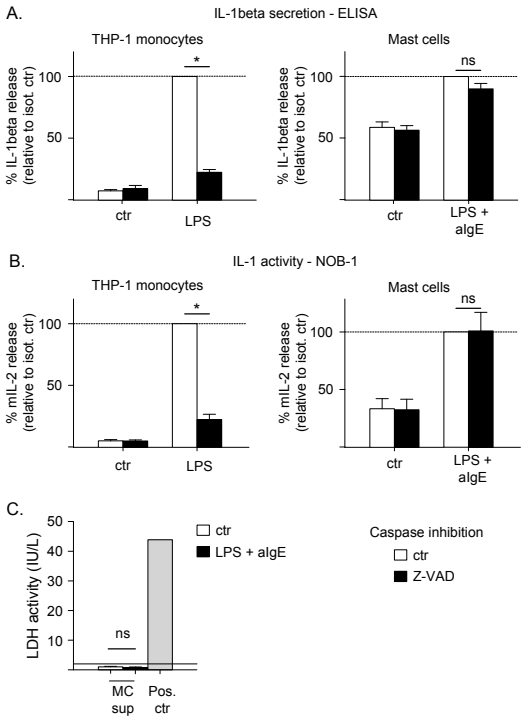


Figure 5 IL-1β release by mast cells is inflammasome-independent. A-B) THP-1 cells and mast cells were left untreated, or stimulated with LPS or LPS + anti-IgE, respectively. Cells were treated with Z-VAD 15 minutes prior to activation. Supernatant was collected after 24 hours. Secretion of IL-1β was measured using ELISA (A). NOB-1 cells were cultured in the presence of cell supernatant, or control medium containing the same stimuli for 24 hours, after which their secretion of mouse IL-2 was measured using ELISA (B). Representative example of THP-1 cell-line is shown (left), and a summary (mean +/- SEM) of 4 different mast cell donors (right). C) Analysis of LDH activity in mast cell supernatant. Mean +/- SEM from 14 independent mast cell donors is shown. Asterisks indicate statistically significant ($p<0.05$) differences analysed using student's T-test. ns indicates no significant differences were found

DISCUSSION

In this study, we show that human mast cells can enhance Th17 responses from memory $CD4^+$ T cells. This is in line with a recent study showing increased IL-22 production by T cells when mast cells presented bacterial antigens.(*2*) Together, these results therefore suggest that human mast cells can contribute significantly to the Th17/Th22 axis.

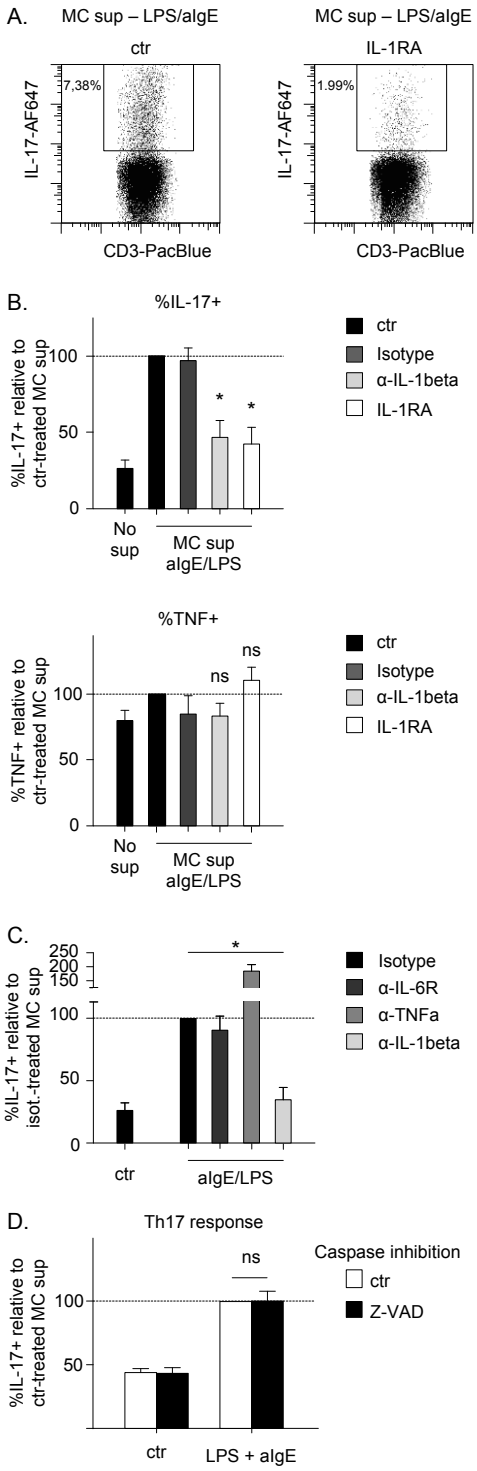


Figure 6. Mast cells induce Th17 responses through inflammasome-independent IL-1β. A-D) Mast cells were left untreated, or stimulated with LPS and anti-IgE. Supernatant was collected after 24 hours. Isolated $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 in the presence of mast cell supernatant or control medium containing the same stimuli. After 5 days, T cells were washed, and stimulated with PMA/ionomycin in the presence of brefeldin A for 5 hours, to analyse their cytokine profile. A-C) Blocking of cytokines in mast cell supernatant was achieved using IL-1RA or blocking antibodies against IL-1β, IL-6R, or TNF-α. Mast cell supernatant or control medium was incubated with these inhibitors prior to addition of the supernatant to the isolated T cells. A) Representative flow cytometry plots of IL-17 production Live T cells were gated as shown in Supplementary Figure 2. B) Summary of inhibition of IL-17 (top) and TNF-α (bottom) in T cells in response to mast cell supernatant untreated (ctr), or treated with anakinra, anti-IL-1β, or isotype control. Mean +/- SEM from 6 independent experiments is shown. C) Summary of inhibition of IL-17 in T cells in response to mast cell supernatant treated with isotype control, anti-IL-6R, anti-TNF-α, or anti-IL-1β. Mean +/- SEM from 3 independent experiments is shown. D) Mast cells were treated with Z-VAD, starting 15 minutes prior to their activation.. Mean +/- SEM from 5 independent experiments is shown. Asterisks indicate statistically significant ($p<0.05$) differences analysed using student's T-test (B) or Two-way ANOVA with Bonferroni posthoc test (C). Ns indicates no significant differences were found, analysed using Two-way ANOVA with Bonferroni posthoc test (D). Percentage $cytokine^+$ T cells that is shown was calculated relative to the % of $cytokine^+$ T cells when treated with supernatant from mast cells activated with LPS + anti-IgE in the presence of isotype control (B, C), or in absence of caspase inhibition (D).

Our results suggest that Th17 expansion by mast cells was induced by IL-1β. IL-1β is a cytokine that is produced in an inactive 31-kDa molecule residing in the cytoplasm, and needs to be cleaved to generate the active 17 kDa molecule, that can be secreted. The most well-known pathway leading to cleavage of IL-1β is the caspase-1 pathway, which is activated upon inflammasome activation. Although inflammasome activation in mast cells can lead to secretion of IL-1β,(*26, 27*) in vivo IL-1β release during sterile inflammation has been shown to be at least partly independent of caspase-1, and mast cell- and neutrophil proteases have been suggested to contribute to secretion of active IL-1β.(*28-32*)

Our results confirm and extend these data, by showing that IL-1β release by human mast cells in response to TLR/FcεRI receptor triggering is caspase-independent as well as that such IL-1-release can lead to boosting of Th17 responses.

A likely candidate for cleavage of pro-IL-1β by mast cells is chymase, one of the granule proteases in mast cells, which has been shown to cleave pro-IL-1β in vitro into an active form of 18kd.(*31*) This would be in line with our observation that IL-1β release by mast cells depends on their degranulation through FcεRI crosslinking. However, as the amounts of IL-1β released in this study were relatively low, we were unable to visualize the mast cell-derived IL-1β by western blot or intracellular staining to analyse the mechanism of cleavage of IL-1β by mast cells. IL-1β is known to be active at extremely low concentrations (<1 pg/mL),(*33*) thereby complicating such analyses.

Our results suggest a novel role of mast cells in driving Th17 responses directly through release of IL-1β in a caspase-independent manner. This is of particular interest in the context of mucosal immunity. Whereas mast cells have been shown to play beneficial roles in protection against bacteria at mucosal surfaces, they also contribute significantly to allergic responses at these same locations.(*34, 35*) Our results suggest that Th17 expansion by mast cells was dependent on FcεRI triggering. Interestingly, allergic responses which are classically considered to be driven by Th2 cells, are now increasingly recognized as also having a Th17 component.(*36*) Our results therefore provide a direct link between IgE-mediated mast cell activation and expansion of Th17 cells in the context of allergic responses.

Together, these studies reveal a role of human mast cells in setting the outcome of T cell responses through the release of inflammasome-independent IL-1β, and suggest a novel contribution of these cells in boosting of immune responses in mucosal barriers by specifically targeting the Th17-axis.

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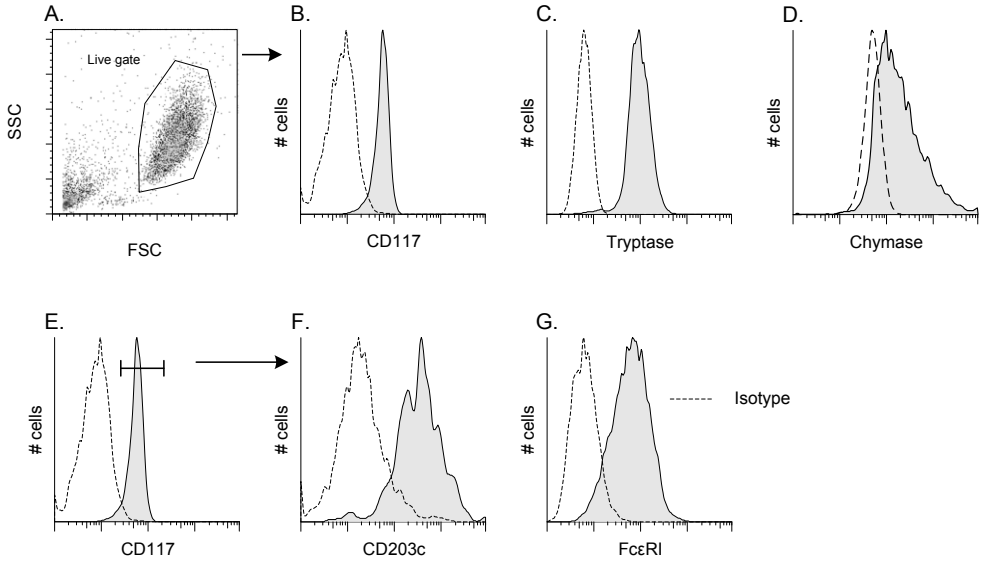
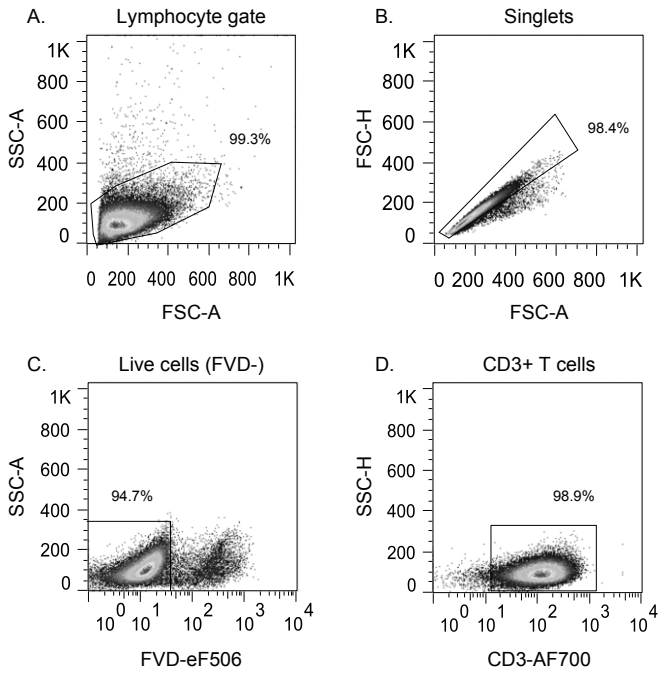
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Characterization of mast cells. Peripheral blood-derived mast cells were cultured from CD34$^+$ stem cells for 6-8 weeks. Mast cells were identified as CD117$^+$ CD203c$^+$ FcεRI$^+$ Tryptase$^+$ cells (>90% pure). A) Gating of live cells (cell debris excluded based on Forward and Sideward scatter). B) CD117 expression of total live cells as gated in A. C) Intracellular Tryptase expression of total live cells. D) Gating of CD117$^+$ mast cells. E,F). Expression of CD203c and FcεRI on CD117$^+$ mast cells as gated in D. Representative FACS plots are shown.



Supplementary Figure 2. T cell gating strategy. Live $CD3^+$ T cells were gated as follows: A) A lymphocyte gate was used based on Forward scatter (FSC) and Side scatter (SSC). B) Single cells were selected based on FSC-area (A) and FSC-height (H). C) Live cells were gated based on negativity for Fixable Viability Dye (FVD) eFluor 506. D) T cells were gated based on CD3 expression.