

IgE-mediated basophil tumour necrosis factor alpha induces matrix metalloproteinase-9 from monocytes

S. Falkencrone¹, L. K. Poulsen², C. Bindselev-Jensen¹, A. Woetmann³, N. Odum³, B. C. Poulsen^{2,4}, L. Blom², B. M. Jensen², B. F. Gibbs⁵, I. M. Yasinska⁵, V. V. Sumbayev⁵ & P. S. Skov^{1,6}

¹Department of Dermatology and Allergy Centre, Odense University Hospital, Odense; ²Allergy Clinic, Copenhagen University Hospital, Gentofte, Denmark; ³Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark; ⁴Laboratory of Immunology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; ⁵Medway School of Pharmacy, University of Kent, Chatham Maritime, UK; ⁶Reflab ApS, Copenhagen, Denmark

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basophils; IgE-mediated activation; monocytes; tissue remodelling; TNF-alpha.

Correspondence

Sidsel Falkencrone, Odense University Hospital, Department of Dermatology and Allergy Centre, Sdr Boulevard 29, 5000 Odense C, Denmark.
Tel.: +45 35457512
Fax: +45 35457506
E-mail: sfal@reflab.dk

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Abstract

Background: IgE-mediated activation of mast cells has been reported to induce the release of tumour necrosis factor alpha (TNF- α), which may display autocrine effects on these cells by inducing the generation of the tissue remodelling protease matrix metalloproteinase-9 (MMP-9). While mast cells and basophils have been shown to express complementary and partially overlapping roles, it is not clear whether a similar IgE/TNF- α /MMP-9 axis exists in the human basophil. The purpose of this study was thus to investigate whether IgE-mediated activation of human basophils induces TNF- α and MMP-9 release.

Methods: Human peripheral blood mononuclear cells (PBMC), isolated basophils and monocytes were stimulated up to 21 h with anti-IgE. Mediator releases were assessed by ELISA, and surface expressions of mediators were detected by flow cytometry. Upregulation of cytokine production was detected by Western blot and polymerase chain reaction (PCR).

Results: IgE-mediated activation of basophils induced the synthesis and release of both TNF- α and MMP-9 from PBMC. In contrast, IgE-mediated activation of purified basophils induced the release and cellular expression of TNF- α but not MMP-9. Isolated monocytes did not release MMP-9 upon anti-IgE stimulation, but MMP-9 release was induced by stimulating monocytes with supernatants from activated basophils, and this release was inhibited by anti-TNF- α neutralizing antibodies.

Conclusion: Our results strongly indicate that human basophils release TNF- α following IgE-dependent activation and that this cytokine subsequently stimulates MMP-9 release from monocytes. These findings support a direct involvement of basophils in inflammation as well as suggesting a role for the basophil in tissue remodelling.

Basophils and mast cells express complementary and partially overlapping roles in acquired and innate immunity but several important distinctions set them apart regarding mediator release and function. Mast cell mediators are usually either preformed/located in granules, such as histamine and prote-

ases, or *de novo* synthesized, such as eicosanoids and cytokines/chemokines (1). However, TNF- α is a cytokine mediator that can be found both preformed and newly synthesized in mast cells and is readily released following Fc ϵ RI cross-linking (2). Mast cell-derived TNF- α has been shown to exert autocrine effects by inducing the upregulation and release of the matrix metalloproteinase-9 (MMP-9) (3–5), indicating that mast cells contribute to tissue remodelling through an IgE/TNF- α /MMP-9 axis.

Basophils share several features with mast cells, including the capacity to generate and store the preformed mediator

Abbreviations

TNF- α , tumour necrosis factor alpha; MMP-9, matrix metalloproteinase-9; IgE, immunoglobulin E; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

histamine in their granules as well as the ability to *de novo* synthesize mediators such as leukotrienes and cytokines including IL-13 and VEGF (6, 7). However, in contrast to mast cells, it has never been demonstrated that basophils express TNF- α as a functional protein, although it has been the subject of investigation by other groups working with basophils (8). Moreover, the ability of basophils to generate MMP-9 is also a controversial subject with contradictory results (4, 9).

Recently, several groups have focused on basophils as players in the innate immune response as well as being involved in adaptive immune responses (10, 11). Additionally, basophils are now widely accepted as being involved in the modulation of inflammation as well as the in the promotion of subsequent tissue repair and angiogenesis (6, 12, 13). Exactly which role basophils play in inflammation still remains an elusive question, but it seems likely that they could contribute to the IgE/TNF- α /MMP-9 axis, as has been demonstrated for mast cells. This would suggest a role for basophils in tissue remodelling, which would be in line with their involvement in inflammation.

The aim of this study was therefore to examine whether IgE-mediated activation of primary human basophils leads to the release and upregulation of TNF- α and MMP-9.

Methods

Cell separation

Peripheral blood mononuclear cells were isolated from anonymized buffy coats obtained from the National University Hospital, Copenhagen, Denmark.

Basophils were isolated from PBMC using the MACS basophil isolation kit II, and monocytes were isolated with the MACS monocyte isolation kit II (Miltenyi Biotech, Bergisch-Gladbach, Germany).

Activation assays

Cell preparations were cultured in RPMI 1640 medium with 1% L-glutamine, 200 mM (100x) and 5% foetal calf serum (FCS) (Invitrogen Corporation, Carlsbad, CA, USA) in a CO₂ incubator. PBMC and basophils were stimulated with 5 ng/ml anti-IgE (KPL, Gaithersburg, MD, USA), and monocytes were stimulated with 0.1 pg/ml TNF- α , 2 pg/ml C5a (both R&D, Minneapolis, MN, USA) or 5 ng/ml anti-IgE. Cellular-expressed MMP-9 or TNF- α levels were measured after cell lysis. Cell viability was examined after 1 and 21 hours incubation by use of Trypan Blue exclusion (14).

For anti-TNF- α experiments, cells were pre-incubated for 1 h with 0.1 ng/ml anti-TNF- α (Remicade, kindly provided by Schering-Plough, Farum, Denmark) before the addition of anti-IgE and kept in medium containing 0.1 ng/ml anti-TNF- α during the remaining 20 h.

In the basophil supernatant experiments, basophils were pulsed for 1 h with anti-IgE and washed twice before incubation for a total of 21 h. Supernatants were then transferred to isolated monocyte samples.

TNF- α , MMP-9 and histamine determinations

TNF- α and MMP-9 levels were measured with commercial ELISA kits (DY911 and DY211, R&D Systems, USA) according to the manufacturer's instructions. Histamine was detected by Reflab ApS (15).

PCR and mRNA

RNA was purified from 500 000 purified basophils per experimental condition (16) using an RNeasy mini kit (Qiagen, Hilden, DE) according to the manufacturer's protocol.

Flow cytometric analysis of surface expression

Binding of mAb to human PBMC was assessed by direct immunofluorescence, and cells were identified as follows: basophils (CD203c, CCR-3), T cells (CCR-3, CD3), monocytes (CD14, CD123), B cells (CD1a, CD19) and dendritic cells (CD83).

Please see Supporting Information for further details on methods.

Statistical analysis

The data were analysed with Shapiro–Wilk test for normality, with a Student's *t*-test for matched pairs or Wilcoxon's test for matched pairs. A *P* value of less than 0.05 was considered statistically significant. In the figures, * denotes *P* < 0.05, ** *P* < 0.01 and *** *P* < 0.001. Medians are shown in the figures as horizontal marks.

Results

Anti-IgE induces TNF- α and MMP-9 release from PBMC

To examine IgE-mediated TNF- α and MMP-9 release from basophils, initial experiments were performed on PBMC incubated with anti-IgE for 21 h.

TNF- α release from 11 PBMC donors was measured in cell supernatants after 1- and 21-h incubation (Fig. 1A). TNF- α levels from cells stimulated for 1 h with anti-IgE (1.0 pg/10⁶ PBMC) were comparable to levels measured from nonstimulated cells (2.1 pg/10⁶ PBMC). IgE-mediated activation of PBMC was found to significantly (*P* < 0.001) induce TNF- α release following stimulation for 21 h (557.0 pg/10⁶ PBMC) compared to nonstimulated controls (12.0 pg/10⁶ PBMC). A similar pattern was observed for cellular contents of TNF- α (Fig. 1B).

As MMP-9 induction by TNF- α is well known from several cell subsets such as mast cells, fibroblasts and monocytes (17, 18), we wished to examine whether anti-IgE, in addition to inducing TNF- α release from PBMC, would also be able to induce MMP-9 release and upregulation in PBMC. The results from 20 donors (Fig. 1C) clearly demonstrated that this was the case and that the pattern of MMP-9 release was similar to the previously observed IgE-mediated release of TNF- α .

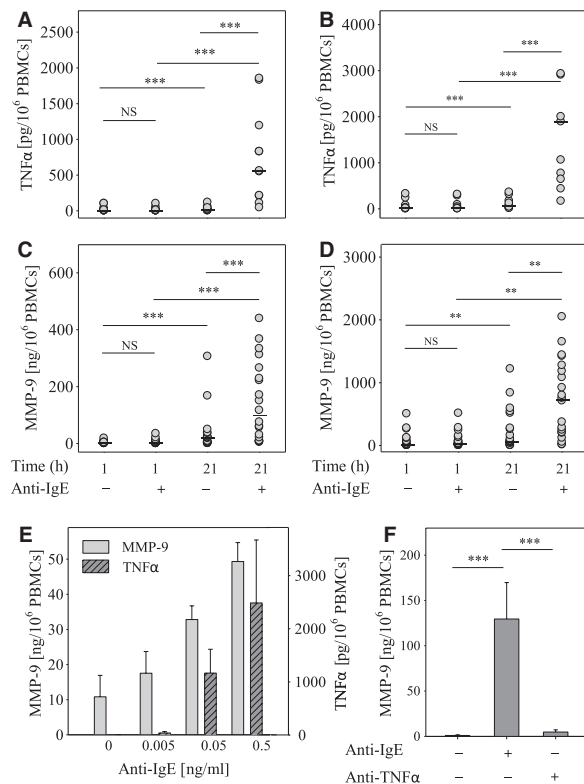


Figure 1 A–F: Release and cellular expression of TNF- α (A–B) and MMP-9 (C–D) from PBMC following 21-h stimulation with anti-IgE. Anti-IgE-induced TNF- α and MMP-9 release was found to be dose dependent (1E), and anti-IgE-induced MMP-9 release was inhibited by anti-TNF- α (1F).

After 1-h incubation, MMP-9 levels were similar in the stimulated and the nonstimulated samples (2 ng/10⁶ PBMC and 1 ng/10⁶ PBMC, respectively). After 21-h incubation, MMP-9 release from cells pulsed with anti-IgE was significantly increased (95.5 ng/10⁶ PBMC, $P < 0.001$) in comparison with nonstimulated cells (18.5 ng/10⁶ PBMC). The same pattern was observed for cellular contents of MMP-9 (Fig. 1D).

Furthermore, we found anti-IgE to induce both MMP-9 and TNF- α release in a dose-dependent manner, with maximal stimulation observed at 0.25–2.5 ng/ml anti-IgE (Fig. 1E).

Anti-TNF- α abolishes IgE-induced MMP-9 release from PBMC

To investigate whether IgE-mediated TNF- α release directly induces MMP-9 release in PBMC, we performed an inhibition experiment where we incubated PBMC with anti-IgE in the presence and absence of anti-TNF- α (Remicade). Here, 1 ng/ml anti-TNF- α significantly inhibited ($P < 0.001$) anti-IgE-induced MMP-9 release from PBMC, where MMP-9 levels from anti-IgE-stimulated PBMC in the presence of anti-TNF- α were equal to those of nonstimulated cells (Fig. 1F). In addition, we observed that these inhibitory effects were dose dependent and statistically significant for

0.1 ng/ml anti-TNF- α (data not shown). This indicates that TNF- α is vital for the induction of MMP-9 release when PBMC are activated through an IgE-dependent mechanism.

Kinetics of TNF- α , MMP-9 and histamine release

The kinetics of TNF- α and MMP-9 release, spontaneous and anti-IgE induced, were also examined (Supporting Information Fig. S1A–F) and showed that while IgE-mediated release of histamine was initiated rapidly and was detected within 1 h, TNF- α and MMP-9 releases were initiated after 10- and 15-h incubation, respectively. These levels increased until 21 h, demonstrating that MMP-9 and TNF- α are not present in the granules but are newly generated mediators.

Anti-IgE does not induce MMP-9 release from purified basophils

Because anti-IgE was observed to cause MMP-9 release from PBMC, this suggested that MMP-9 may have originated from IgE-mediated basophil activation. The experiments were therefore repeated using highly purified basophils from six donors. However, 1-h stimulation with anti-IgE did not induce any MMP-9 release from purified basophils (0.07 ng/10⁶ basophils) when compared to nonstimulated controls (0.07 ng/10⁶ basophils). Similarly, we also failed to detect any increase in MMP-9 release from either stimulated (0.7 ng/10⁶ basophils) or unstimulated cells (0.1 ng/10⁶ basophils) after 21-h incubation. These experiments clearly demonstrate that MMP-9 is not expressed or released after anti-IgE stimulation (Fig. 2A). Additionally, the lack of MMP-9 was confirmed by PCR, Western blot and zymography (data not shown).

Anti-IgE induces TNF- α production in purified basophils

To identify the source of TNF- α from PBMC, purified human basophils (97–99% pure) from 12 donors were stimulated with or without anti-IgE (Fig. 2B).

Spontaneous TNF- α release in supernatants was low (6.0 pg/10⁶ basophils) after 1 h but increased significantly ($P < 0.001$) after 21-h incubation (962.1 pg/10⁶ basophils) as shown in Fig. 2B. Anti-IgE-induced TNF- α release after 1-h pulsing (12.0 pg/10⁶ basophils) did not differ from corresponding spontaneous TNF- α release. However, after 21-h stimulation, anti-IgE-induced TNF- α release was significantly increased (2928.5 pg/10⁶ basophils, $P < 0.001$). The same pattern was observed for cellular expression of TNF- α measured in cell lysates with cellular levels of TNF- α to vary between donors in the range of 2–10 times the release levels (Supporting Information Fig. S2A).

IgE-mediated release of TNF- α from basophils was observed to be dose dependent following the release pattern of the established basophil mediator histamine (Fig. 2C) and similar to what has been described for basophil-derived IL-13 and IL-4 (7, 17). Induction of TNF- α from basophils by IgE-mediated activation was further confirmed by TNF- α mRNA measurements in four donors of highly purified ba-

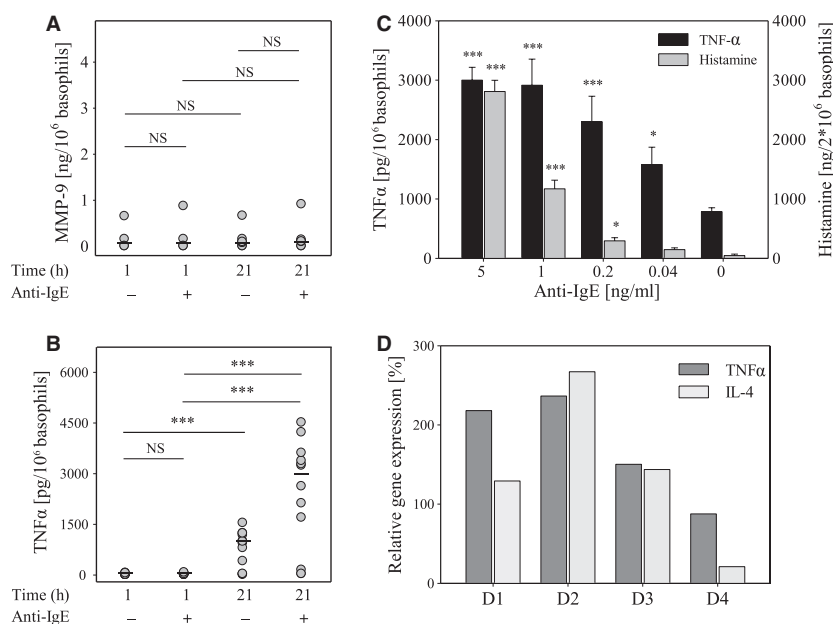


Figure 2 A–D: IgE-mediated activation of isolated basophils did not induce MMP-9 release (2A) – please note the scaling on the y-axis. Anti-IgE stimulation of isolated basophils induced TNF- α release (2B) and histamine release in a dose-dependent manner (2C). IgE-

mediated activation of isolated basophils also increased relative gene expression of TNF- α and IL-4 mRNA. Results are shown as % expression when compared to nonstimulated controls, which are defined as 100% (2D).

sophils (98%) as shown in Fig. 2D. When comparing relative gene expressions in unstimulated basophils to that of anti-IgE-stimulated basophils, IgE-mediated stimulation induced a relative gene expression of 150–236% in three of the four donors, while one donor was nonresponding.

To confirm that basophils express TNF- α in a biologically active 17-kDa monomeric form (because 51-kDa inactive trimers of TNF- α can also be detected by ELISA assays), Western blotting was performed on lysed pellets from anti-IgE-stimulated basophils, confirming that TNF- α was released in the active monomer form with a molecular weight of 17 kDa (Supporting Information Fig. S2B). Additionally, our preliminary immunocytochemistry data support the presence of intracellular TNF- α in basophils (Fig. S3).

IgE-mediated activation of PBMC induces MMP-9 release from monocytes through basophil-derived TNF- α

To determine the cellular source of MMP-9 in anti-IgE-stimulated PBMC, flow cytometry was performed with antibodies against MMP-9 and cell-specific markers for basophils, B cells, plasma cells, NK cells, monocytes and T cells. As seen in Fig. 3A, only CD14⁺ monocytes appeared to upregulate surface-bound MMP-9 upon 21-h IgE-mediated activation of PBMC; intracellular staining was found to give similar results.

To exclude a direct effect of anti-IgE on monocytes when stimulating PBMC with anti-IgE, highly purified monocytes ($\geq 98\%$) were incubated with anti-IgE for 21 h after which no significant MMP-9 release or upregulation could be detected. Monocytes were also incubated with TNF- α and

C5a, both of which induced MMP-9 release, thereby confirming the functionality of the isolated monocytes and supporting our findings that IgE-mediated activation alone does not induce MMP-9 release from monocytes (Fig. 3B).

Basophil supernatant induces MMP-9 from monocytes

Based on our findings that anti-IgE stimulates basophil TNF- α release, we asked whether basophil-derived TNF- α could trigger monocytes to release MMP-9, because TNF- α is known to induce MMP-9 from these cells.

To investigate this, we pulsed highly purified basophils ($\geq 98\%$) with anti-IgE for 1 h and removed anti-IgE by thorough washing before further incubation. After subsequent 21 h of incubation, fractions of basophil supernatants, previously shown not to contain MMP-9, were transferred to purified monocytes, which were then incubated for 21 h before MMP-9 detection.

Monocytes released MMP-9 when stimulated with supernatant from both anti-IgE-stimulated and nonstimulated basophils (Fig. 4A). However, supernatants from IgE-activated basophils more readily induced MMP-9 release from monocytes than supernatants from basophils incubated in the absence of anti-IgE.

Based on our previous results, demonstrating inhibition of MMP-9 release by anti-TNF- α and MMP-9 release from monocytes due to basophil supernatants, we isolated monocytes and pre-incubated them with anti-TNF- α before stimulation with the same basophil supernatants generated for the results shown in Fig. 4A. We found that monocyte-

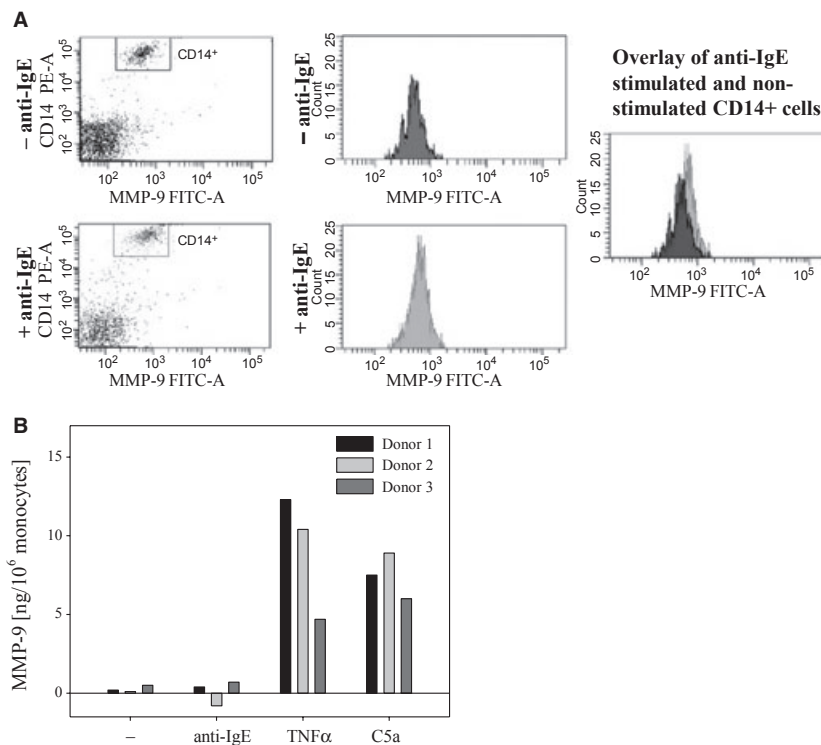


Figure 3 A–B: PBMC stimulated for 21 h with anti-IgE were analysed for surface expression of MMP-9. Only CD14+ cells showed surface expression of MMP-9, which was increased by anti-IgE stimulation for 21 h (3A). To investigate whether monocytes could

react directly to anti-IgE, MMP-9 release was measured from three donors of isolated monocytes stimulated 21 h with TNF- α , C5a or anti-IgE. Monocytes were found to induce MMP-9 release upon TNF- α and C5a but not anti-IgE stimulation (3B).

derived MMP-9 release, induced by basophil supernatants stimulated with/without anti-IgE, was inhibited by anti-TNF- α (Fig. 4B), demonstrating that basophil supernatants induce MMP-9 release from monocytes through basophil-derived TNF- α .

Discussion

It has been known for several years that IgE-mediated activation of human mast cells causes TNF- α release that can, by autocrine mechanisms, induce the release of prestored MMP-9 (2, 3). The purpose of the present study was to investigate whether basophils could release either preformed or newly synthesized TNF- α and MMP-9 by a mechanism similar to mast cells. There are controversies in the literature regarding basophils and the presence or absence of MMP-9 in this cell (4, 9), and there are no published data concerning basophils and TNF- α (8). Cell migration is known to be facilitated by metalloproteinases such as MMP-9 (19), and one could speculate that IgE-mediated basophil activation induces MMP-9 release through an IgE/TNF- α /MMP-9 axis as seen in mast cells. This would facilitate further basophil migrations into tissues affected by allergic inflammation, which is possibly supported by reports showing that MMP-9 levels are increased in allergic inflammatory diseases such as asthma and urticaria (2, 20) where basophils accumulate in lung and skin tissues, respectively (12, 21).

Our initial experiments were performed to examine the release of TNF- α and MMP-9 from mixed leucocytes (PBMC) caused by IgE-dependent stimulation. Our results showed that levels of TNF- α and MMP-9 in unstimulated PBMC were close to the minimum detection level but that anti-IgE dose dependently increased levels of both mediators after 21-h incubation. However, we did not detect preformed TNF- α or MMP-9 in PBMC and kinetics experiments showed that both mediators were released after 10- to 15-h incubation following initial anti-IgE stimulation. It is worth mentioning that while ~10–20% of PBMC donors are nonresponders in regard to histamine release, we did not find this to be the case when investigating TNF- α and MMP-9 release from PBMC.

Anti-TNF- α completely abolished IgE-mediated MMP-9 release from PBMC. This is in line with previous observations in mast cells as described by Baram et al. (3) and supports our hypothesis that basophils act through an IgE/TNF- α /MMP-9 axis.

After establishing that PBMC release TNF- α and MMP-9 upon IgE-mediated activation, we investigated TNF- α release from purified basophils as well as basophil-derived TNF- α in a mixed cell populations. Our results clearly demonstrated that in 12 individual donors, IgE-mediated activation of highly purified basophils led to TNF- α release measured in the supernatant after 21 h. In a paper by Chen et al. (22), it was suggested that IgD-mediated activation of basophils could potentially cause TNF- α release, but this was not fur-

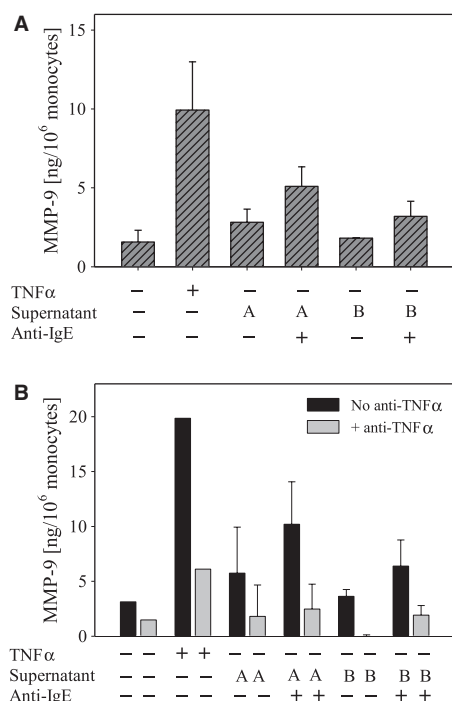


Figure 4 A–B: (A) Isolated monocytes were incubated with supernatants from purified basophils (basophil donor A/B) that had been pulsed with or without anti-IgE ($\pm$). TNF- α stimulation of monocytes was included as control of functional MMP-9 release by TNF- α , and levels of MMP-9 in monocyte supernatants were measured after 21 h. Results are shown from three representative donors of monocytes stimulated with supernatant from two different basophil donors. (B) Monocytes were pre-incubated 1 h with or without anti-TNF- α (Remicade) and stimulated with anti-IgE-pulsed basophil supernatant for 21 h to investigate inhibition of basophil-induced MMP-9 release from monocytes caused by anti-TNF- α . Results are shown from two representative monocyte donors stimulated (A/B) with supernatants from two different basophil donors (A/B) that were pulsed with or without anti-IgE ($\pm$) (4B). Please note that the error bars represent intradonor variation in the monocyte samples.

ther elaborated. To the best of our knowledge, this is the first comprehensive study demonstrating that basophils are a source of TNF- α . We attempted to develop a flow cytometric protocol to demonstrate intracellular expression of TNF- α in isolated basophils and in basophils as part of a mixed cell population (PBMC). However, in line with other groups involved in basophil research, we were not able to demonstrate this, possibly owing to technical problems associated with low expression of TNF- α in individual cells. Despite this, intracellular TNF- α was clearly detected in purified basophil lysates by ELISA, Western blotting and immunocytochemistry. The 2% contamination by CD3⁺ T cells and unidentified cell types was considered as a potential source of the TNF- α release, but this was rejected based on the fact that this would require release levels of 10 μ g TNF- α /10⁶ T cells, levels that to our knowledge never have been demonstrated for any cell type.

To identify the cellular origin of MMP-9 released in a mixed cell population, we investigated IgE-mediated MMP-9 release from highly purified basophils and observed that they did not express or release MMP-9. MMP-9 could not be detected in either supernatants or intracellularly in resting or IgE-activated basophils after 21 h incubation. The ability of basophils to generate MMP-9 was investigated in two previous publications (4, 9). Hayashi et al. showed a lack of MMP-9 expression or release from basophils by gelatin zymography and immunocytochemistry, whereas Suzukawa et al. concluded that basophils are a source of MMP-9 in a transmigration study. The reasons for these discrepancies have not been clarified but may have arisen as a result of the use of other basophil markers than the 'classic' basophil markers such as CCR-3, CD63 and CD203c (23).

To identify the source of MMP-9 in PBMC, we assessed both intracellular and surface MMP-9 expression in different cell subsets with/without IgE-mediated activation. We found that only CD14⁺ cells (monocytes) showed an increase in MMP-9 expression upon IgE-mediated activation of PBMC.

Because monocytes can bind IgE (24), we investigated whether anti-IgE could induce TNF- α release from purified monocytes but did not find any release of this cytokine. TNF- α did, however, induce MMP-9 release from monocytes, as previously described (18, 25), and it was therefore tempting to speculate that IgE-mediated TNF- α release from basophils triggered MMP-9 release from monocytes. Indeed, supernatants obtained from basophils after pulsing with/without anti-IgE were able to induce MMP-9 release from isolated monocytes.

To support the concept that TNF- α is the cytokine in basophil supernatants responsible for causing monocyte MMP-9 release, we performed inhibition experiments using anti-TNF- α antibodies (Remicade). Supernatants from IgE-pulsed basophils were incubated with purified monocytes that had been pre-incubated with anti-TNF- α , and we found an inhibition of MMP-9 release from monocytes of up to 80%. The inhibition was not complete indicating that other cytokines or substances from basophils could be involved in monocyte activation.

Our study demonstrates for the first time that IgE-mediated activation of basophils induces TNF- α release. While it is well known that human mast cells, besides tryptase and chymase, possess preformed proteases such as MMP-9 (3–5), this does not seem to be the case for human basophils. However, our results indicate that these cells may act by proxy by inducing MMP-9 from monocytes. This suggests an important regulatory role for basophils, in which IgE-mediated activation may lead to cytokine-driven tissue remodelling and chronic inflammation.

Acknowledgments

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Conflict of interest

The authors have no conflict of interest to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. A–F: Kinetics of TNF- α , MMP-9 and inhibition of MMP-9 by anti-TNF- α over a time course of 21 h. Results are shown from three donors and normalised as % as release towards anti-IgE induced release at 21 h. Results given as ng/10⁶ PBMCs for MMP-9 and pg/10⁶ PBMCs for TNF- α . TNF- α release from PBMCs during 21 h with or without anti-IgE (Fig. 1A–B). MMP-9 release from PBMCs during 21 h with or without anti-IgE (Fig. 1C–D). MMP-9 release

from PBMCs pre-incubated with or without anti-TNF- α before stimulation with anti-IgE for 21 h (Fig. 1E–F).

Figure S2. A–B. Cellular expression of TNF- α in pellets of purified basophils with or without anti-IgE stimulation (Fig. S2A). Additionally, basophil cell pellets were analysed with Western Blotting after 2 h stimulation with anti-IgE, and the molecular weight of detected TNF- α was found to be 17 kDa, indicating that basophils release TNF- α in a bio-active monomer form. Lysate from a THP-1 monocyte cell line indicates basal TNF- α expression as a positive control (Fig. S2B).

Figure S3. Intracellular expression of TNF- α and IL-4 in cytopins of purified basophils with and without anti-IgE stimulation. Preliminary data obtained from two separate basophil donors. Results are shown following 2 h incubation $\pm$ anti-IgE. Bottom panel shows unstimulated basophils stained with May–Grünwald stain.

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Online supplementary for:

IgE-mediated basophil tumor necrosis factor alpha induces matrix metalloproteinase-9 from monocytes

Sidsel Falkencrone¹, Lars K. Poulsen², Carsten Bindslev-Jensen¹, Anders Woetmann³, Niels Odum³, Britta C. Poulsen^{2,4}, Lars Blom², Bettina M. Jensen², Bernhard F. Gibbs⁵, Inna M. Yasinska⁵, Vadim V. Sumbayev⁵ & Per Stahl Skov^{1,6}.

¹Department of Dermatology and Allergy Centre, Odense University Hospital, Denmark; ²Allergy Clinic, Copenhagen University Hospital Gentofte, Gentofte, Denmark; ³ Department of International Health, Immunology and Microbiology, University of Copenhagen, Denmark; ⁴Laboratory of Immunology, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; ⁵Medway School of Pharmacy, University of Kent, Chatham Maritime, United Kingdom; and ⁶Reflab ApS, Copenhagen, Denmark.

METHODS FOR ONLINE SUPPLEMENTARY

Cell separation

PBMCs were isolated from anonymized buffy coats obtained from the National University Hospital, Clinical Immunology - Blood Bank, Copenhagen, Denmark, by standard density gradient centrifugation with Lymphoprep (Axis Shield, Norway). PBMCs were harvested from the interface, washed twice in isotonic (0.9%) NaCl₂ solution and kept at 37°C in RPMI medium until use.

23 Basophils were isolated from PBMCs by magnetic sorting using the negative selection MACS
24 Basophil Isolation Kit II, and monocytes isolated using the MACS Monocyte Isolation Kit II (both
25 kits from Miltenyi Biotech, Germany). Purifications were performed according to the
26 manufacturer's instructions. Purity of isolated basophils was confirmed by Alcian Blue staining as
27 well as by flow cytometry, and the fraction discarded if below 97%.

28 Basophils and other leukocytes were stained for surface expression and identified as follows:
29 Basophils (CD203c, CCR-3), T-cells (CCR-3, CD3), monocytes (CD14, CD123), B-cells (CD1a,
30 CD19) and dendritic cells (CD83). In all three purified basophil preparations, contamination was
31 found to be below 2% and comprised mainly of CD3+ T-cells and, to a minor part (0.5 %), of un-
32 identified cells that did not express CCR-3, CD1a, CD14, CD19, CD83, CD123 or CD203c.

33

34 Monocytes were investigated by flow cytometry as CD14+ and CD123+ cells, and cell fractions
35 were discarded if purity was below 95%. Purity of monocyte fractions was further investigated by
36 histamine measurements in lysed cell samples to ensure that no basophils were present in monocyte
37 preparations.

38

39 Cell viability for all cell-subsets was examined after 1 and 21 hours incubation by use of Trypan
40 Blue exclusion and viability was found to be $\geq 95\%$.

41

42 Functionality of basophils in regards to IgE-mediated activation before and after 21 hour incubation
43 was addressed by measuring IgE-mediated histamine release from basophils in a mixed cell
44 suspension (PBMCs) that had been incubated 1 or 21 hours with 5% fetal calf serum and 1% L-

45 Glutamine. The IgE-mediated histamine was found to be comparable regardless of the incubation
46 period.

47

48 TNF- α , MMP-9 and histamine determinations

49 Quantitative enzyme-linked immunosorbent assays (ELISAs) was used to analyze culture
50 supernatants and lysed cell pellets for TNF- α and MMP-9 with commercial ELISA kits (DY911
51 and DY211, R&D Systems, USA) according to the manufacturer's instructions. Protein
52 concentrations were determined by extrapolation from a standard curve generated by serial dilution
53 of the standard supplied with the ELISA kits.

54 Histamine was detected by Reflab ApS in accordance to the company's accredited glass fibre
55 method.

56

57 Time-course experiments

58 Measurements of TNF- α and MMP-9 were performed in samples incubated up to 21 hours.
59 Incubation for 42 hours was performed (data not shown) but since no additional TNF- α or MMP-9
60 was released after 21 hour incubation, 21 hours was chosen as the endpoint for our experiments.

61

62 PCR and mRNA

63 RNA was purified from 500,000 purified basophils per experimental condition⁽¹⁵⁾ using an RNeasy
64 mini kit (Qiagen, Hilden, DE) according to the manufacturer's protocol. RNA concentrations were
65 measured using a SmartSpec 3000 (BioRad, Berkeley, CA, USA) spectrophotometer. cDNA was

66 synthesized from 0.1 µg total RNA using Superscript II reverse transcriptase (Invitrogen) and stored
67 at -20 °C. qRT-PCR analysis of mRNA expression was performed using Taqman Universal PCR
68 master mix (Applied Biosystems, Carlsbad, CA, USA). Gene expressions were determined using
69 primer assays forβ-actin (401846) and TNF-α (Hs99999043_m1) (Applied Biosystems). The
70 mixture was run in triplicates on a StepOnePlus running StepOne Software v2.1 (Applied
71 Biosystems). Data were analyzed using the $\Delta\Delta$ cycle threshold (CT) method.

72 Values representing TNF-α or IL-4 mRNA levels were normalized against a house-keeping gene
73 (actin) and then against mRNA levels of non-stimulated basophils.

74

75 Western Blot

76 TNF-α was determined by Western blot analysis of basophil lysates. After 2 hours incubation with
77 or without anti-IgE, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and
78 lysed in 200 µl of Western Blot lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.5%
79 Nonidet-40, 1 mM PMSF, pH 8.0). After centrifugation (13000 rpm, 15 min) the protein content in
80 the supernatants was analysed. Lysates from a THP-1 cell line, which is known to produced TNF-α,
81 was also analysed as a positive control.

82

83 40 µg of protein was added to the same volume of 2× sample buffer (125 mM Tris-HCl, 2%
84 sodium dodecyl sulfate (SDS), 10% glycerine, 1 mM dithiothreitol (DTT), 0.002% bromphenol
85 blue, pH 6.9) and boiled for 5 min. Proteins were resolved on 12.5% SDS-polyacrylamide gels and
86 blotted to nitrocellulose membranes. Molecular weights were calibrated in proportion to the running
87 distance of rainbow markers. Mouse anti-TNF-α antibody was added and incubated for 60 min at

88 room temperature. Afterwards, nitrocellulose membranes were washed five times for 15 min and
89 subjected to incubation with Li-Cor (Lincoln, Nebraska USA) goat secondary antibodies conjugated
90 with fluorescent dyes for 60 min according to the manufacturer's protocol. β -actin staining was used
91 to confirm equal protein loading as described previously. The Western blot data for TNF- α protein
92 were subjected to a quantitative analysis using Odyssey software and values were normalised
93 against respective actin bands.

94

95 Immunocytochemistry

96 Purified basophils were incubated with or without anti-IgE for 2 or 18 hours. Cells were then
97 washed once, resuspended in HEPES-buffered Tyrode's solution (containing 1% BSA), and then
98 centrifuged onto glass slides using a Shandon Cytospin® 4 Cytocentrifuge (2min; 500rpm). Slides
99 were then air dried at 4°C and permeabilized using -20°C methanol for 10 min. After blocking for
100 2h, cells were incubated overnight with primary antibodies to either TNF- α (R&D Systems, USA),
101 IL-4 (Biolegend) or isotype IgG controls (DAKO). Slides were then washed with PBS 5x for 15
102 min in total. FITC-conjugated anti-mouse secondary antibodies (1/250 dil.) were then added for 2h
103 followed by washing and fluorescence microscopy.

104

105 **SUPPLEMENTARY FIGURE LEGENDS**

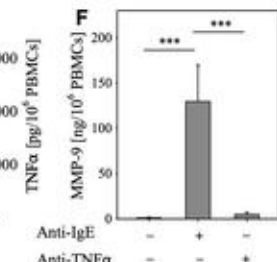
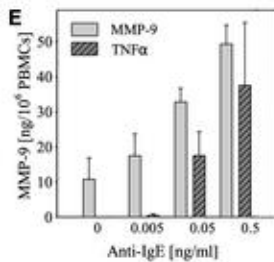
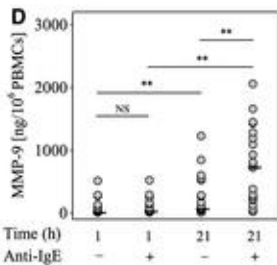
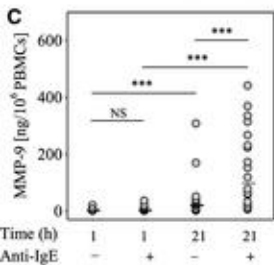
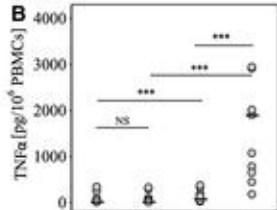
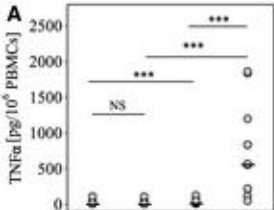
106 Fig. S1a-f: Kinetics of TNF- α , MMP-9 and inhibition of MMP-9 by anti-TNF- α over a time course
107 of 21 hours. Results are shown from three donors and normalised as % as release towards anti-IgE
108 induced release at 21 hour. Results given as ng/10⁶ PBMCs for MMP-9 and pg/10⁶ PBMCs for
109 TNF- α . TNF- α release from PBMCs during 21 hour with or without anti-IgE (Fig. 1a-b). MMP-9
110 release from PBMCs during 21 hour with or without anti-IgE (Fig. 1c-d). MMP-9 release from
111 PBMCs pre-incubated with or without anti-TNF- α before stimulation with anti-IgE for 21 hours
112 (Fig. 1e-f).

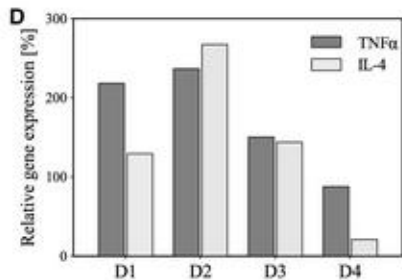
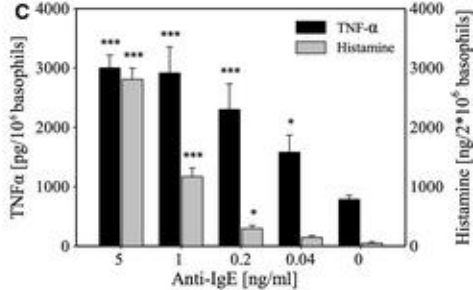
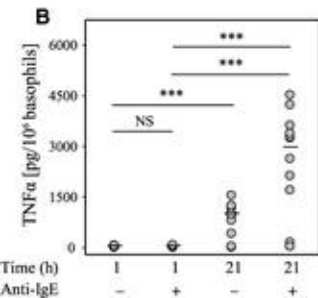
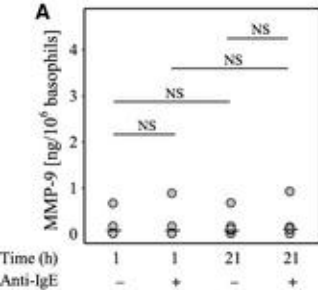
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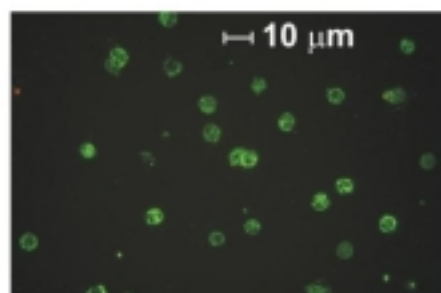
114 Fig. S2a-b. Cellular expression of TNF- α in pellets of purified basophils with or without anti-IgE
115 stimulation (Fig. S2a). Additionally, basophil cell pellets were analysed with Western Blotting after
116 2 hour stimulation with anti-IgE, and the molecular weight of detected TNF- α was found to be 17
117 kDa, indicating that basophils release TNF- α in a bio-active monomer form. Lysate from a THP-1
118 monocyte cell line indicates basal TNF- α expression as a positive control (Fig. S2b).

119

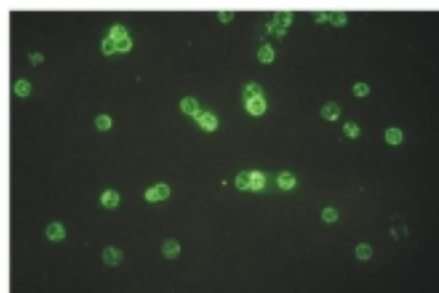
120 Fig. S3. Intracellular expression of TNF- α and IL-4 in cytopins of purified basophils with and
121 without anti-IgE stimulation. Preliminary data obtained from 2 separate basophil donors. Results are
122 shown following 2 hour incubation $\pm$ anti-IgE. Bottom panel shows unstimulated basophils stained
123 with May-Grünwald stain.



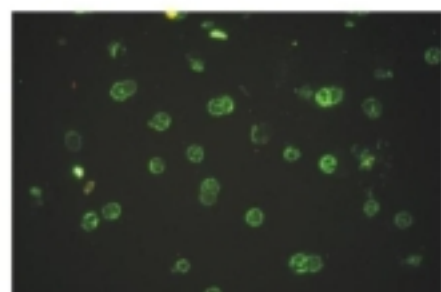




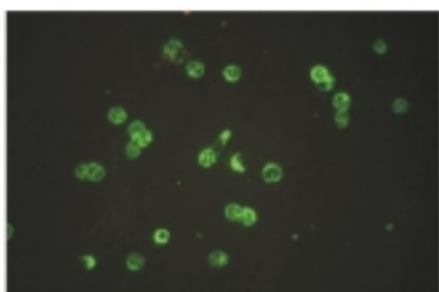
Ctrl + anti-TNF- α



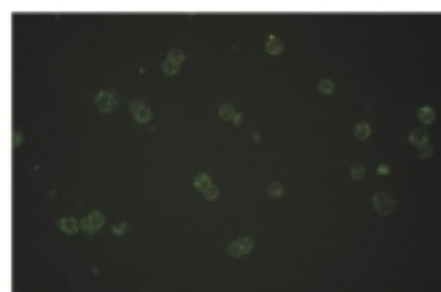
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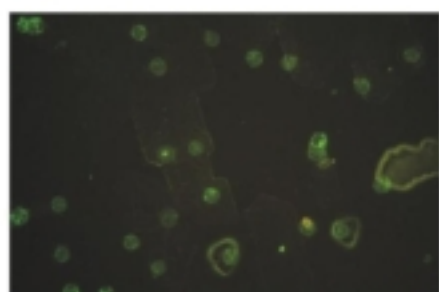
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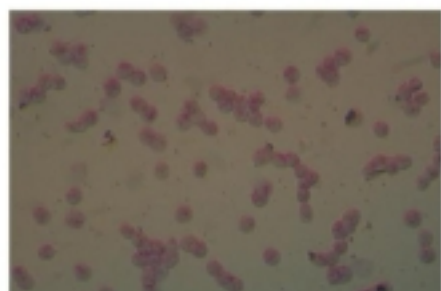
algE + anti-IL-4



Ctrl + Iso



algE + Iso



MG