In vitro and in vivo modulation of human T lymphocytes from allergic asthmatic subjects
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Citation for published version (APA):
Huijsen, F. L. (2001). In vitro and in vivo modulation of human T lymphocytes from allergic asthmatic subjects

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Products from human mast cell line (HMC-1) cells enhance the production of IFN-γ by CD8⁺ and CD4⁺ T cells

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submitted for publication
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Abstract

In patients with allergic asthma, T cell cytokines are implicated in the regulation of the local inflammation in the airways. The ability of sensitised mast cells to release mediators and cytokines early upon allergen stimulation makes them important candidates for local immunoregulation. We have studied the effects of human mast cells on T cells with the use of the human mast cell line HMC-1. We showed that activated human mast cells or their soluble products induced and enhanced the IFN-γ production by T-cells up to about 60 fold. The production of IL-4 was hardly affected and that of IL-5 was slightly enhanced. The enhancement of IFN-γ production was induced both in polyclonal CD4⁺ and CD8⁺ T cells and in CD4⁺ and CD8⁺ T-cell clones. Further characterisation of the factors involved demonstrated a molecular mass above 30 kDa. Our results implicate that by this mechanism mast cells may account for a negative feedback system locally downregulating allergen-induced Th2 responses via IFN-γ production by the T cells.

Introduction

In patients with allergic asthma, T cells are involved in regulation of local inflammation in the airways. High numbers of T cells in biopsies and in the bronchoalveolar lavage fluid from patients show signs of activation [1,2]. These T cells produce cytokines like IL-2, IFN-γ, IL-4, IL-5 and IL-13, which may play a pivotal role in airway inflammation [3,4]. The Th2 cytokines IL-4 and IL-5 are important regulators in IgE mediated allergic reactions and they are abundantly expressed in the airways in severe and in symptomatic asthma [5,6]. In mild asthma, and shortly after experimental allergen exposure the Th1 cytokine IFN-γ may be highly expressed as well [7-10].

The migration of T cells into airway mucosal tissue and airway lining fluid, and the local activation of T cells are regulated by cytokines, mediators and cell-cell interactions. In this respect mast cells may play in important role either by direct cell-cell contact or by the release of mediators and cytokines [11]. Mast cells contain factors chemotactic for T cells (IL-16, lymphotactin) [11], which are released upon activation. In mast cell-deficient mice there is no influx of mononuclear cells into the mucosal tissue after local IgE-mediated reactions [12]. Furthermore, mast-cell mediators like histamine and prostaglandins, and mast-cell derived cytokines modulate T-cell proliferation and cytokine production [11,13-15]. Interestingly, mast cells derived from the nasal mucosa of patients with allergic rhinitis
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may release IL-4 by which they may contribute to ongoing IgE production and allergic reactions [16].

We have earlier reported on the effects of the mast-cell mediator histamine on human T cells. Histamine enhanced or inhibited T-cell proliferation and cytokine production dependent on the cell type studied and conditions applied [14,15]. To obtain information on the influence of the whole spectrum of mast-cell products on T cells we have extended our studies and used the human mast cell line HMC-1 as a model for mast cells [17]. As a source of T cells we used purified polyclonal human CD4$^+$ and CD8$^+$ T-cell populations as well as T-cell clones. Our most prominent finding in these studies is a strong stimulation by mast cells of the production of IFN-γ by T cells. These results suggest that mast cells may be important regulators in determining whether T cells differentiate to Th1 and Th2 cells.

Material and Methods

Subjects
Lymphocytes from non-smoking healthy adults and adult patients with allergic asthma were studied. The healthy subjects had no airway complaints and they had no IgE antibodies to a panel of common aeroallergens. Asthma was defined according to the American Thoracic Society criteria [18] and included a history of recurrent episodes of wheezing, chest tightness and dyspnoea and a normal lung function between asthma attacks. The patients had bronchial hyperreactivity (the concentration of histamine causing a fall in the FEV$_1$ of more than 20% was below 8 mg/ml) [19], and they had IgE antibodies to house dust mite. The patients had not experienced an exacerbation of asthma during at least two months preceding the study. The patients did not use anti-inflammatory medication during at least 6 weeks preceding the study, in particular they did not use corticosteroids. The study was approved by the Medical Ethics Committee of the Academic Medical Center.

Mast cells and supernatants
Human Mast Cell-line cells, HMC-1, were from Dr. J. Butterfield [17]. Cells were kept in culture medium I: Iscove's Modified Dulbecco's Medium (IMDM; GIBCO-BRL, Paisley, UK) supplemented with 5% heat-inactivated fetal calf serum (BioWhittaker, Verviers, Belgium), 2 x 10$^{-4}$ M β-mercaptoethanol (Merck, Munich, Germany), 50 µg/ml transferrin (Boehringer Mannheim, Mannheim, Germany), 180 µg/ml L-glutamine (Merck), 1.25 µg/ml
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Amphotericin B (Fungizone; GIBCO, Grand Island NY), penicillin (100 U/ml, Gist Brocades, Delft, The Netherlands) and streptomycin (100 µg/ml, GIBCO). Cell cultures were incubated at 37°C in humidified air containing 5% CO₂. Before using mast cells in the assays they were prepared as follows. HMC-1 cells were washed with Earle's Balanced Salts supplemented with Tris buffer (GIBCO) containing 2% fetal clone serum (Hyclone, Logan, Utah, USA). Cells were then resuspended at a concentration of 0.4 to 1.6 x 10⁶/ml in culture medium II (IMDM supplemented with 10% heat-inactivated pooled human serum (BioWhittaker, Walkersville MD), 2 x 10⁻⁵ M β-mercaptoethanol (Merck), 2 mM Sodium-pyruvate (Merck), penicillin (100 U/ml, Gist Brocades) and streptomycin (100 µg/ml, GIBCO). The cells were incubated for 30 minutes at 37°C without or with stimulation (Phorbol Myristate Acetate (Sigma, St. Louis, MO, USA), final concentration 10 ng/ml, and calcium ionophore A23187 (Sigma), final concentration 10 µg/ml). Next, the cells were collected by centrifugation and resuspended in washing medium at 0.4 to 1.6 x 10⁶/ml. To prevent their proliferation during the subsequent assays, cells were irradiated by 3000 rad of gamma-irradiation at 0°C. Subsequently, the cells were centrifuged once more and resuspended in culture medium II at 0.4 to 1.6 x 10⁶/ml. Cells were incubated for 24 h at 37°C in humidified air containing 5% CO₂. Supernatants were removed after centrifugation of the cells and were stored at -20°C. For some experiments, supernatants were divided into fractions enriched for <30 kDa molecules and >30 kDa molecules, respectively. To achieve this, samples were fractionated on centrifprep tubes (Amicon, Beverly MA, USA) by centrifugation for 30 min at 3000 x g.

Polyclonal T lymphocytes and T-lymphocyte clones

Blood was obtained by venepuncture and collected in sodium heparin. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden; d = 1.078) centrifugation ofuffy coats from healthy subjects. Purified CD8⁺ T-cell populations were obtained by negative selection. PBMC were incubated with saturating amounts of anti-CD4, anti-CD16, anti-CD19 and anti-CD56 mAb (all from CLB Sanquin Blood Supply Foundation, Amsterdam, The Netherlands) at 4°C for 30 minutes. After washing away excess mAb, magnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with sheep anti-mouse IgG were added. The magnetic beads were concentrated by a Dynal Magnetic Particle Concentrator. The remaining negatively selected cells were subjected once more to the same procedure to obtain further depletion. The negatively selected CD8⁺ T-cell population was >84% CD8⁺ and contained <0.15% CD4⁺ cells as determined by flow cytometry analysis. The CD8⁺ T cells were immediately used or kept in culture in
Effects of mast cells on CD8⁺ and CD4⁺ T lymphocytes

medium III (culture medium II plus irradiated PBMC as feeder cells, IL-2 (20 U/ml, Lymfocult, Biotest, Dreiech, Germany)) and phyto-hemagglutinin (PHA, Murex Diagnostics Ltd, Dartford, UK). Cell cultures were incubated at 37°C in humidified air containing 5% CO₂.

Polyclonal CD45RA⁺ and CD45R0⁺ cells were obtained from a freshly purified polyclonal CD8⁺ T-cell population. Cells were divided into two portions and incubated at 4°C for 30 minutes with saturating amounts of anti-CD45RA-FITC or anti-CD45R0 (Beckton Dickinson (BD), San José CA, USA), respectively. The latter were subsequently incubated with Goat-anti-Mouse F(ab)₂-FITC (CLB) for 30 minutes at 4°C. Cells were then sorted by FACS Star Plus (BD). Both FITC-positive and FITC-negative fractions were collected. Experiments were performed with the negatively selected fractions; in the fraction where CD45R0⁺ (both bright and dull staining) cells were removed the CD45R0⁻ (and CD45RA⁺) naive cells were left over, in the other fraction CD45RA⁺ (bright and dull staining) cells were removed leaving the CD45RA⁻ (and CD45R0⁺) memory cells. Naive and memory cells were not cultured any further but used directly in the assays.

T-lymphocyte clones were prepared as described before [8,20]. Briefly, cloning was performed using direct limiting dilution with the use of an automated cell deposition unit coupled to the FACStar Plus (BD). Clones were generated in the culture medium III using coated anti-CD3 mAb 16A9 as a stimulus. Positive clones were transferred to plates, mostly after 2-3 weeks and treated as follows below. For the experiments described in this study, we selected clones with a Th0 phenotype, producing both IFN-γ and IL-4. Several clones produced IL-5 as well.

At weekly intervals, polyclonal lines and clones were restimulated with PHA, IL-2 and irradiated PBMC and cultured at 0.4 to 0.8 x 10⁶ cells/ml at 37°C. The experiments were performed at day 7 after the restimulation. Three days before performing the experiments with the polyclonal T cells or the T-cell clones, the culture medium was replaced by medium II. Just before the experiments, the cells were collected, washed with washing medium and resuspended at 0.4 x 10⁶/ml in medium II.

**T-cell proliferation and cytokine production**

Roundbottom plates (96-well, Costar, Cambridge, MA, USA) were coated in quintuplicate overnight with graded quantities of anti-CD3 mAb (16A9) diluted in Phosphate Buffered Saline (PBS) supplemented with penicillin (100 U/ml, Gist Brocades) and streptomycin (100 µg/ml, GIBCO), after which the plates were washed three times with PBS. T-cells (40,000 cells/well) were added. Where indicated, either 40,000 irradiated HMC-1 cells (resting or
stimulated), or 40,000 irradiated PBMC, or (fractionated) supernatants, or other substances were added to the wells. Cultures were incubated for the indicated time-points at 37°C in humidified air containing 5% CO₂. For assay of cytokine production, the plates were centrifuged, supernatants of 5 parallel wells were pooled and stored at -20°C until the day of assay. T-cell proliferation was assessed in parallel by measuring [³H]-thymidine incorporation after the addition of 0.2 μCi [³H]-thymidin (Amersham, Buckinghamshire, UK) before finishing the culture. Proliferation data are shown as the mean of quintuplicates. In some experiments T cells were stimulated with anti-CD3 mAb T3/4 plus 1XE (CLB) plus anti CD28 (CLB), or with Concanavalin A (ConA, ICN, Biomed).

**Assays for cytokines and mediators**

IFN-γ, IL-5 and IL-6 contents were measured by ELISA [15,21,22]. Briefly, plates coated with mAb anti-IFN-γ (MD2), anti-IL-5 (TRFK-5) or anti-IL-6 (mIL-6-16M) were incubated with sample together with an excess of biotinylated mAb anti-IFN-γ (MD1), anti-IL-5 (mAb7) or anti-IL-6 (sIL-6). Plates were washed and subsequently incubated with streptavidin-horseradish peroxidase (CLB) and finally developed using tetramethylbenzidine (Merck) as a substrate. The lower limits of detection were 62.5 pg IFN-γ/ml, 47 pg IL-5/ml and 3 pg IL-6/ml. Levels of IL-4, IL-8, PGE₂, TxB2 were assessed using commercially available kits (CLB, CLB, Amersham, and Amersham, respectively). Lower limits of detection were 1.95 pg IL-4/ml, 1.02 pg IL-8/ml, 10 pg PGE₂/ml, and 3.6 pg TxB2/ml.

**Reverse transcription PCR for IFN-γ**

Polyclonal CD8⁺ T-cells (1 x 10⁶ per well) were cultured for 0 min, 30 min, 1 h, 2 h and 4 h without or with (stimulated) mast cells in the presence of anti-CD3. Total RNA was extracted and isolated using TRIzol Reagent (GIBCO) according to manufacturer's description. First strand cDNA was synthesized from total RNA using Superscript II RNAses H reverse-transcriptase (GIBCO). PCR amplification was carried out in PCR buffer (Eurogentec; final volume 50 μl) containing MgCl₂ (Eurogentec), dNTP (Pharmacia), Taq DNA polymerase (Goldstar) and β2m- or IFN-γ-specific primers (Eurogentec). We co-amplified PQA1, a multi-specific internal control [23]. The mixture was covered by mineral oil (Sigma) and denatured at 95°C for 5 minutes. The PCR reaction comprised 35 cycles (94°C, 60°C and 72°C, each for 1 minute). PCR products were separated by electrophoresis on a 2% agarose gel and visualized with UV light after ethidium bromide staining. The intensity of the bands was analysed by EagleEye (Stratagene, Amsterdam, the Netherlands). For calculations those samples were used in which intensity of the bands of the T-cell
Effects of mast cells on CD8+ and CD4+ T lymphocytes

product differed not more than factor 2 from the intensity of the PQA1 product. Absolute amounts of IFN-γ mRNA were calculated using the known amount of the PQA1 product and were related to the absolute amounts of β2-microglobulin which were calculated in the same way as IFN-γ.

Statistics

The Mann Whitney U (MWU) test was applied to analyse differences between groups. The Wilcoxon matched pair signed rank test was used to evaluate differences between paired samples. Two-sided p values below 0.05 were considered statistically significant.

Results

Products from HMC-1 cells

To verify the stimulation of the HMC-1 mast cells the production of several cytokines and mediators was measured. The following were found to be produced by resting and by PMA plus A23187 stimulated mast cells: IL-8, 49 ± 58 pg/ml and 1020 ± 259 pg/ml (mean ± standard deviation, n = 5), respectively; prostaglandinE2, 208 ± 83 and 278 ± 81 pg/ml (n = 2), respectively; IL-6, 10 and 32 pg/ml, respectively; LTB4, 223 and 541 pg/ml, respectively; and TxB2, 611 and 855 pg/ml, respectively. IFN-γ, IL-4, IL-5 or IL-12 could not be detected in the supernatants.

Effects of HMC-1 cells on polyclonal CD8+ T cells and polyclonal CD4+ T cells

Resting HMC-1 cells enhanced the proliferation of anti-CD3 stimulated polyclonal CD8+ T cells 1.5 ± 0.2 fold (mean ± sem, n = 8 experiments). Irradiated PBMC were used as a control for the effects of adding extra cells and resulted in a 1.7 fold increase. Stimulated mast cells led to a decrease of proliferation to 38 ± 7% of control (mean ± sem, n = 8 experiments). The irradiated HMC-1 cells and PBMC alone showed very low 3H-thymidine incorporation. HMC-1 mast cell-line cells had essentially the same effects on polyclonal CD4+ cells. The proliferation of CD4+ T cells was 1.7 fold increased by resting mast cells, and 1.8 fold by resting PBMC as control. Stimulated mast cells decreased proliferation to 24% of control.
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Figure 1. IFN-γ production at 24 h by anti-CD3 stimulated polyclonal CD8+ T lymphocytes (mean ± SD). Where indicated different amounts of resting HMC-1 cells or stimulated HMC-1 cells were added to the wells.

Figure 1 shows a representative experiment with polyclonal CD8+ T cells. When incubated with anti-CD3 the cells produced 200 ± 71 (mean ± sd) pg IFN-γ/ml. The addition of resting HMC-1 cells enhanced IFN-γ production to 700 ± 122 pg/ml. Stimulated mast cells increased IFN-γ production up to 24.7 ± 2.1 ng/ml in a dose-dependent manner. The results from 6 experiments with polyclonal CD4+ T-cell populations and 13 experiments with polyclonal CD8+ T-cell populations obtained from 3 different donors are shown in Table 1. For CD4+ T cells the ratio (IFN-γ production in the presence of anti-CD3 plus stimulated mast cells) / (IFN-γ production in the presence of anti-CD3) was 59.2 ± 68 (mean ± sd) and for CD8+ T cells this ratio was 11.8 ± 3.2. The increase of IFN-γ production by stimulated mast cells was significantly higher than that by resting mast cells or resting PBMC (p<0.01).

To check if the above mentioned effects of stimulated mast cells were mast cell-specific, PBMC were treated in exactly the same way as the HMC-1 cells. Part of the PBMC were stimulated with PMA and A23187, the other part incubated without the stimulus. Also the irradiation and washing procedures were performed in precisely the same way. The addition of resting PBMC thus treated increased the T-cell proliferation 1.7 fold. The addition of stimulated PBMC thus treated did not affect proliferation as compared to the proliferation without added PBMC. Both resting and stimulated PBMC caused a 1.9 to 2.0-fold increase of IFN-γ production. Thus, the effects of stimulated PBMC were qualitatively and quantitatively different from those of stimulated HMC-1 cells.
Effect of mast cells on CD8+ and CD4+ T lymphocytes

Table 1. IFN-γ production at 24 h by anti-CD3 stimulated polyclonal CD4+ and polyclonal CD8+ T lymphocytes. Where indicated resting HMC-1 cells, stimulated HMC-1 cells or resting PBMC were added to the wells.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>CD4 (n = 6)</th>
<th>CD8 (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3 alone</td>
<td>0.61 ± 0.7*</td>
<td>1.74 ± 2.1</td>
</tr>
<tr>
<td>+ Resting HMC-1</td>
<td>1.31 ± 1.4</td>
<td>3.4 ± 3.4</td>
</tr>
<tr>
<td>+ Stimulated HMC-1</td>
<td>15.2 ± 13.3</td>
<td>12.4 ± 11</td>
</tr>
<tr>
<td>+ Resting PBMC</td>
<td>1.0 ± 0.8</td>
<td>3.3 ± 3.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratios</th>
<th>CD4 (n = 6)</th>
<th>CD8 (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting HMC-1/anti-CD3</td>
<td>2.55 ± 0.9**</td>
<td>2.51 ± 0.4</td>
</tr>
<tr>
<td>Stimulated HMC-1/anti-CD3</td>
<td>59.2 ± 68†</td>
<td>11.8 ± 3.2†</td>
</tr>
<tr>
<td>Resting PBMC/anti-CD3</td>
<td>2.35 ± 1.0</td>
<td>1.88 ± 0.3#</td>
</tr>
</tbody>
</table>

*, ng IFN-γ/ml, mean ± 1 standard deviation. **, mean ± sem. †, higher than the ratio resting HMC-1/anti-CD3, p<0.001; higher than the ratio resting PBMC/anti-CD3, p<0.01; #, lower than resting HMC-1/anti-CD3, p<0.02.

To substantiate the effect of mast cells on IFN-γ production we measured the effect of mast cells on IFN-γ mRNA. Polyclonal CD8+ T cells were cultured up to 4 hours without or with (stimulated) mast cells in the presence of anti-CD3. Table 2 shows that already at 30 min IFN-γ mRNA was found to be much higher in the T-cell culture to which the activated mast cells were added than in the cultures without the mast cells. IFN-γ mRNA increased up to at least 4 h of culture. At this time-point levels of IFN-γ mRNA in the T-cell culture with resting mast cells were similar to anti-CD3 stimulation alone. IFN-γ protein was measured in all culture supernatants and was normally below 48 pg/ml. When stimulated mast cells were added onto the T-cell cultures the IFN-γ production was increased to 88 pg/ml and 1 ng/ml at 2h and 4h, respectively. The results of these experiments show that stimulated mast cells induced a rapid increase of IFN-γ mRNA.

The production of IL-4 by the polyclonal CD8+ T cells was low (4.5 pg/ml). It was not increased by resting HMC-1 cells, and it was only slightly increased (to 5.2 pg/ml) by irradiated PBMC. Stimulated mast cells increased IL-4 production to 10 pg/ml. IL-5 could not be detected in these T-cell supernatants nor in those from polyclonal CD4+ T cells. Neither the irradiated mast cells nor the irradiated PBMC produced detectable levels of IFN-γ, IL-4 or IL-5.
Table 2. Quantities of IFN-γ mRNA (in fg/100 fg β2microglobulin mRNA) present in the cultures of anti-CD3 stimulated polyclonal CD8+ T lymphocytes (1 x 10⁶ cells) without or with (stimulated) HMC-1 cells (4 x 10⁶ cells).

| Time (h) | Polyclonal + resting + stimulated CD8+ T-cells HMC-1 cells HMC-1 cells |
|----------|-------------------|------------------|------------------|
| 0        | 0.042             | ND               | < 0.02           |
| 0.5      | 0.022             | ND               | 0.478            |
| 1        | 0.056             | ND               | 1.356            |
| 2        | 0.109             | ND               | 5.162            |
| 4        | 0.601             | 0.596            | 14.780           |

**CD8+CD45RA+ and CD8+CD45RO+ cells**

CD45RO+ T cells have a higher IFN-γ production capacity than the CD45RA+ T cells [24]. To test whether the increase of IFN-γ production might be related to a differentiation of T cells, the effect of stimulated mast cells was studied with purified CD8+CD45RA+ and CD8+CD45RO+ T cells. Anti-CD3 stimulated naive cells did not produce detectable amounts of IFN-γ. Stimulated (but not resting) mast cells induced the naive cells to produce 550 pg IFN-γ/ml. Anti-CD3 stimulated memory cells produced 393 pg IFN-γ/ml. Resting mast cells led to increased IFN-γ production (4.4-fold). Stimulated mast cells increased IFN-γ production 12.2-fold. Thus, the effects exerted by HMC-1 cells were similar for both naive and memory CD8+ T-cells.

**Effect of mast cell supernatant and individual products**

Mast cells were stimulated as described in methods and incubated for 24 h at 37°C. Supernatant was collected and added in a 1:2 or 1:4 dilution to polyclonal CD8+ T cells. The 1:2 diluted supernatant decreased proliferation from 11,000 cpm to 6,000 cpm and increased IFN-γ production from 3 ng/ml to 14 ng/ml. When the supernatant was diluted to 1:4, both proliferation and IFN-γ production were as with anti-CD3 alone, illustrating that the effect of the supernatant was a dose-dependent effect. This experiment was repeated 3 times with essentially the same results. Supernatants from not-stimulated mast cells and supernatants from irradiated PBMC that had been stimulated like the mast cells had no effects.

As HMC-1 cells were shown to produce histamine, TNF-α, IL-1β, IL-8 and PGE₂ [17,25], and as IL-12 and IL-18 are known IFN-γ enhancers [26], we added different doses of these mediators separately to CD8+ T-cell cultures. Though some of them had a small stimulatory effect on IFN-γ production, none of the mediators could account for the increase...
Effects of mast cells on CD8+ and CD4+ T lymphocytes

of IFN-γ production we found when T cells were cultured with the stimulated HMC-1 cells themselves.

Next, experiments were performed in which mast cell supernatants and medium were divided into fractions enriched for <30 kDa molecules and >30 kDa molecules, respectively. These enriched fractions were added to polyclonal CD8+ T cells and IFN-γ production was measured. Upon anti-CD3 stimulation polyclonal CD8+ cells produced 680 pg/ml IFN-γ. The >30 kDa enriched fraction of the supernatant of stimulated mast cells increased IFN-γ production 23 times, whereas the low molecular weight fraction had no effect.

**Different kinds of T-cell stimuli**

To learn more about the effects of mast cells on T cells in combination with different T-cell stimuli, the following experiment was conducted. Polyclonal CD8+ T-cells were cultured in medium II, supernatant of resting HMC-1 mast cells or supernatant of stimulated HMC-1 mast cells. When no T-cell stimulus was applied polyclonal T-cells did not produce detectable levels of IFN-γ and supernatant of resting mast cells had no effect (Table 3).

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>None</th>
<th>Supernatant of resting mast cells</th>
<th>Supernatant of stimulated mast cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
<td>6.2</td>
</tr>
<tr>
<td>Anti-CD3 + anti-CD28</td>
<td>0.28</td>
<td>0.36</td>
<td>12.5</td>
</tr>
<tr>
<td>PHA (0.5 μg/ml)</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
<td>5.2</td>
</tr>
<tr>
<td>PMA (1 ng/ml)</td>
<td>&lt;0.06</td>
<td>&lt;0.06</td>
<td>12.5</td>
</tr>
<tr>
<td>Ionomycin (1 μg/ml)</td>
<td>0.51</td>
<td>0.39</td>
<td>2.3</td>
</tr>
<tr>
<td>PMA (1 ng/ml) + ionomycin (1 μg/ml)</td>
<td>13.8</td>
<td>13.0</td>
<td>4.7</td>
</tr>
<tr>
<td>ConA (0.5 μg/ml)</td>
<td>&lt;0.06</td>
<td>0.09</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Supernatant of stimulated mast cells led to an induction of IFN-γ production of 6.2 ng/ml. Similar results were seen for the stimuli PHA, PMA and ConA. Soluble anti-CD3 + soluble anti-CD28, and ionomycin induced low levels of IFN-γ production (0.3 ng/ml and 0.5 ng/ml, respectively). Then, the supernatant of stimulated mast cells increased IFN-γ
production to 12.5 ng/ml and 4.7 ng/ml, respectively, and supernatant of resting mast cells hardly affected IFN-γ production. When T cells were stimulated by PMA + ionomycin, there was already a high production of IFN-γ (13.8 ng/ml). Here, resting mast cells had no effect and the supernatant of stimulated mast cells inhibited IFN-γ production to 34% of control.

**Effects of HMC-1 cells on CD4^+ and CD8^+ T-cell clones**

The effect of HMC-1 cells was tested on homogeneous and further defined cell populations, CD8^+ T-cell clones and CD4^+ T-cell clones obtained from healthy and asthmatic subjects (Figures 2-4). The anti-CD3 induced production of IFN-γ by CD8^+ T cell clones was higher than that by CD4^+ T-cell clones, both for clones from healthy subjects (mean ng/ml ± sem: 0.95 ± 0.25 and 0.1 ± 0.02, respectively) as for those from patients with allergic asthma (2.02 ± 0.44 and 0.46 ± 0.23, respectively) (p<0.01, MWU test). Resting mast cells led to no or small increases of IFN-γ production (Figure 2). Stimulated HMC-1 cells caused additional increases of IFN-γ production for all clones studied, except in some of the CD4^+ T-cell clones from the asthmatic subjects (Figure 2). CD8^+ and CD4^+ T-cell clones from healthy subjects had similar IFN-γ production in the presence of stimulated mast cells. The CD8^+ T-cell clones from allergic asthma patients had still higher IFN-γ production (6.1 ± 1.9 ng/ml) than the CD4^+ T-cell clones from the patients (1.2 ± 0.5 ng/ml) (p<0.01, MWU test). Furthermore, the fold increase of IFN-γ production by stimulated mast cells was higher in healthy subjects (mean: 44) than in asthma patients (mean 4.4) (p<0.03, MWU test). This resulted in slightly higher IFN-γ production in the presence of stimulated mast cells by clones from healthy subjects (10.8 ± 3.5 ng/ml) than by clones from asthma patients (3.4 ± 1.2 ng/ml) (p=0.08, MWU test).

IL-4 production was mostly decreased after addition of resting mast cells (Figures 3A and 3B). In most of the clones IL-4 production was not affected by stimulated mast cells. Mast cells had similar effects on IL-4 production by T-cell clones from healthy subjects.

Several T-cell clones produced IL-5. The production of IL-5 by CD8^+ T-cell clones from healthy subjects was not affected by resting mast cells. Stimulated mast cells increased the IL-5 production by 4 out of 5 CD4^+ T-cell clones from asthma patients (Figure 4).
Figure 2. (A) IFN-γ production at 24 h by anti-CD3 stimulated clonal CD8⁺ T lymphocytes. Clones were obtained from the peripheral blood of 3 healthy control subjects. Where indicated resting HMC-1 cells or stimulated HMC-1 cells were added to the wells. (B) As figure 2A but clonal CD4⁺ T lymphocytes from 3 healthy control subjects. (C) As figure 2A but clonal CD8⁺ T lymphocytes from 4 allergic asthmatic subjects. (D) As figure 2C but clonal CD4⁺ T lymphocytes from 2 allergic asthmatic subjects. The mean stimulation of IFN-γ production by stimulated mast cells was 44-fold in CD8⁺ T cells from healthy subjects which was significantly higher than the mean stimulation (4.4-fold) in CD8⁺ T cells clones from asthma patients (p < 0.03, MWU test).
Figure 3. (A) IL-4 production at 24 h by anti-CD3 stimulated clonal CD8⁺ T lymphocytes. Clones were obtained from the peripheral blood of 3 allergic asthmatic subjects. Where indicated resting HMC-1 cells or stimulated HMC-1 cells were added to the wells. (B) As figure 3A but clonal CD4⁺ T lymphocytes from 1 allergic asthmatic subject.

Figure 4. (A) IL-5 production at 24 h by anti-CD3 stimulated clonal CD8⁺ T lymphocytes. Clones were obtained from the peripheral blood of 3 healthy subjects. Where indicated resting HMC-1 cells or stimulated HMC-1 cells were added to the wells. (B) As figure 4A but clonal CD4⁺ T lymphocytes.
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**IL-4 recovery**

Because mast cells were found to be able to degrade [27] or bind IL-4 we performed an experiment to check if this was the case for the HMC-1 cells as well. Therefore, recovery of IL-4 was measured in the supernatant of resting mast cells. When 100 pg/ml IL-4 was added to a culture of $0.4 \times 10^6$ and $0.8 \times 10^6$ resting mast cells/ml the IL-4 recovered after 24 h was 80% and 58%, respectively, of the control situation where IL-4 was incubated without mast cells. Thus, there appears to occur some degradation of IL-4 or binding of IL-4 onto the mast cells.

**Discussion**

We have studied the effects of human mast cells on T cells with the use of the human mast cell line HMC-1. This is an immature mast cell line with tryptase levels being 1/10th of normal human lung mast cells [28,29]. Cells lack the gamma-chain of the FcεI receptor and have a mutation in the c-kit receptor. Therefore we stimulated the mast cells with PMA and calcium-ionophore A23187. Here we show that soluble products from stimulated mast cells modulate human T-cell responses in that they can induce and increase especially the IFN-γ mRNA and IFN-γ protein production by CD4* and CD8* T cells, both in polyclonal T-cell populations and in T-cell clones. The increase of the IFN-γ production was already detectable at 2h after stimulation of the cells. The production of IL-4 was hardly affected and there was a slight enhancement of IL-5 production in several T-cell clones. The experiments with the T-cell clones showed that the effects of mast cells are direct effects on T cells.

Our findings are in line with those in an animal model reported by Tkaczyk et al. [30]. In contrast to this Huels et al. [31] found that co-culturing of anti-DNP-IgE sensitized bone marrow derived mast cells with naive spleen CD4* T cells led to a decrease of IFN-γ production by the T cells, while at the same time IL-4 was induced. They could (partially) block these effects by adding anti-IL-4 to the co-cultures. When we added IL-4 to the polyclonal CD8* T-cells we also found a (dose-dependent) decrease of IFN-γ production (data not shown). This indicates that in case of the HMC-1 cells factors other than IL-4 may be involved in the regulation of IFN-γ production. We have tested other possible HMC-1 cell products like histamine, PGE₂, PGD₂, TNF-α, IL-1α, IL-1β and IL-8, and they did not induce large stimulation of IFN-γ production when added separately. Furthermore, we have found that the IFN-γ enhancing cytokines IL-12 and IL-18 could not account for the increase
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of IFN-γ production that was induced when T cells were cultured with the (supernatant of) stimulated HMC-1 cells. Thus, it might be that other factors than the above-mentioned are involved, or maybe a combination of factors is necessary to exert the effects. From the experiments with the mast-cell supernatants (Table 3) it became clear that stimulation of mast cells is necessary to obtain the effects. Resting mast cells did not produce the stimulus.

Information on the molecular weight of the factor(s) responsible for the increase of IFN-γ production was obtained by experiments in which we separated culture medium and (resting and stimulated) mast-cell supernatants into fractions enriched for either < 30 kDa molecules or > 30 kDa molecules. The IFN-γ enhancing capacity was exclusively found in the > 30 kDa fraction of the stimulated mast-cell supernatant. We were not able to identify the components which are responsible for the increase in IFN-γ by the T cells.

Because human mast cells were found to release IL-4 protein [32], they are commonly thought to favour differentiation of T-cells to the Th2 phenotype [33-35]. However, a study with human lung mast cells (obtained from normal lung) failed to detect release of IL-4 protein [36] and another study suggested that these cells are unable to induce the class switch to IgE in B-lymphocytes in the absence of exogenous IL-4 [37]. Nasal mast cells of allergic rhinitics stimulated with allergen, on the contrary, were able to release low levels of IL-4 and high levels of IL-13, and they could induce IgE synthesis [16]. IL-4 mRNA was higher in bronchial mucosal tissue from allergic asthma patients than from non-atopic controls [5], and part of the IL-4 protein was associated with mast cells [6,33]. Thus, mast cells from allergic subjects may be different from those from healthy controls in that they are able to promote the Th2-response, whereas mast cells from healthy subjects may induce a Th1-response. It would be interesting to know whether this results from intrinsic differences between mast cells from those populations or it results from divergent differentiation of mast cells in allergic subjects versus healthy persons. The HMC-1 cell line cells represent an immature type mast cell and did not produce IL-4 under the conditions applied. In contrast, some degradation or binding of IL-4 was observed.

The study of the CD4+ and CD8+ T-cell clones revealed that the CD8+ T-cell clones produced higher amounts of IFN-γ when incubated with anti-CD3 alone as compared to the CD4+ clones. This is in line with the finding [24] that differentiated CD8+ T cells are able to produce higher amounts of IFN-γ than CD4+ T cells. The T-cell clones from the healthy subjects were more responsive to the activated mast cells than those from the patients. Earlier we showed that the IFN-γ production by T-cell clones from allergic subjects was not enhanced by histamine in contrast to those from healthy controls [15]. Thus, the T cells from patients with allergic asthma appeared to respond with lower IFN-γ production upon
stimulation than those from healthy subjects, like it has been found for allergic subjects by several other groups as well.

IFN-γ may act as a proinflammatory cytokine. It may activate alveolar macrophages thereby possibly counteracting the suppressive effect of alveolar macrophages on T cells. Furthermore, IFN-γ may induce and enhance the expression of cell adhesion molecules on leukocytes and airway epithelial cells [38]. It may be hypothesised that when there is activation of mast cells in the lungs in vivo, the products released result in a rapid enhancement of the production of IFN-γ by local T lymphocytes. In this way T-cell products may contribute to the late phase bronchusobstructive reaction that often occurs after exposure of allergic asthmatic patients to aeroallergens. In fact, high levels of IFN-γ and an increase of IFN-γ-positive T cells [39] have been demonstrated in bronchoalveolar lavage fluid shortly after local allergen exposure.

Furthermore, IFN-γ is well-recognised for its inhibitory activity on the differentiation towards Th2 cells [40]. Some studies in mice and rats indicate a role for CD8+ T cells in the in vivo downregulation of IgE production by the production of IFN-γ [41]. A similar mechanism may be operative in human cells, though the reactions involved may be more complicated than just IFN-γ production [41]. In men, in vivo it has been found that asthmatic subjects with markedly increased CD8+ T cell numbers in bronchoalveolar lavage fluid are less likely to develop a late asthmatic reaction [42,43] which also points at a potential suppressive role for the CD8+ T cell. Thus, a long lasting or a chronic effect of an increased IFN-γ expression in the lungs may be the induction of a relative skewing to Th1 reactions, whereas an acute effect of IFN-γ release after allergen contact could be proinflammatory.

A possible implication of our findings might be that together with the decreased proliferation the increased levels of IFN-γ over IL-4 could account for a negative feedback system by which mast cells locally downregulate allergen-induced Th2 responses via the CD8+ T cells, and may be to a lesser extent also by the CD4+ T cells [44]. A lower responsiveness of T cells in allergic subjects in this respect may be one of the underlying causes for the development of the allergic symptoms. However, whether HMC-1 cells resemble normal mast cells and to what extent normal mast cells differ from mast cells from allergic subjects remains to be investigated. Thus, further studies are required using isolated lung mast cells instead of the mast cell line.
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Acknowledgements

The authors wish to thank Dr. J.H. Butterfield (Mayo Clinic, Rochester, Minnesota, USA) for providing the HMC-1 cell line. We thank Dr. R.A.W. van Lier for providing the mAb 16A9, Dr. A. Snijders for measuring IL-12 p40 and Dr. J. Meenan for the measurements of TxB2 and LTB4. C.H. van Oven and R. Hoebe are gratefully acknowledged for their assistance with the FACS Star Plus and S. van Wissen for assistance with T-cell clone culture experiments.

This study was financially supported by a grant from the Netherlands Asthma Foundation (grant no. 93.46) and the Stichting Astma Bestrijding.

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