

## Products from human mast cell line cells enhance the production of interferon- $\gamma$ by CD8<sup>+</sup> and CD4<sup>+</sup> T cells

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### SUMMARY

In patients with allergic asthma, T-cell cytokines are implicated in the regulation of the local inflammation in the airways. The ability of sensitized 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 interferon- $\gamma$  (IFN- $\gamma$ ) production by T cells up to about 60-fold. The production of interleukin (IL)-4 was hardly affected and that of IL-5 was slightly enhanced. The enhancement of IFN- $\gamma$  production was induced both in polyclonal CD4<sup>+</sup> and CD8<sup>+</sup> T cells and in CD4<sup>+</sup> and CD8<sup>+</sup> T-cell clones. Further characterization of the factors involved demonstrated a molecular mass above 30 000. Our results implicate that by this mechanism mast cells may account for a negative feedback system locally down-regulating allergen-induced T helper 2 responses via IFN- $\gamma$  production by the T cells.

### INTRODUCTION

T lymphocytes are involved in regulation of immunologic and inflammatory reactions in immunoglobulin E (IgE)-allergic subjects. In patients with allergic asthma, for example, high numbers of T cells in biopsies and in the bronchoalveolar lavage fluid from patients show signs of activation.<sup>1,2</sup> These T cells produce cytokines such as interleukin (IL)-2, IL-4, IL-5, IL-13 and interferon- $\gamma$  (IFN- $\gamma$ ), which may play a pivotal role in airway inflammation.<sup>3,4</sup> The T helper type 2 (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.<sup>5,6</sup> In mild asthma, and shortly after experimental allergen exposure the Th1 cytokine IFN- $\gamma$  may be highly expressed as well.<sup>7–10</sup>

The migration of T cells into local inflammatory sites, and the local activation of T cells are regulated by cytokines,

mediators and cell–cell interactions. In this respect mast cells may play an important role either by direct cell–cell contact or by the release of mediators and cytokines.<sup>11</sup> Mast cells contain factors chemotactic for T cells (IL-16, lymphotactin),<sup>11</sup> 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.<sup>12</sup> Furthermore, mast-cell mediators like histamine and prostaglandins, and mast-cell derived cytokines modulate T-cell proliferation and cytokine production.<sup>11,13–15</sup> Interestingly, mast cells derived from the nasal mucosa of patients with allergic rhinitis may release IL-4 by which they may contribute to ongoing IgE production and allergic reactions.<sup>16</sup>

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.<sup>14,15</sup> 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.<sup>17</sup> This cell line has many characteristics in common with native mast cells,<sup>18</sup> and has been applied in a number of studies on characteristics of mast cells. As a source of T cells we used

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purified polyclonal human CD4<sup>+</sup> and CD8<sup>+</sup> 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- $\gamma$  by T cells. These results suggest that mast cells may be important regulators in determining whether T cells differentiate to Th1 and Th2 cells.

## MATERIALS AND METHODS

### Subjects

Lymphocytes from non-smoking healthy adults and from allergic subjects were studied. The healthy subjects had no IgE antibodies to a panel of common aeroallergens. The allergic subjects had IgE antibodies to house dust mite; they 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.<sup>17</sup> Cells were kept in culture medium I as described.<sup>19</sup> 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 Life Technologies Ltd, Paisley, UK) containing 2% fetal clone serum (Hyclone, Logan, UT), and resuspended at a concentration of  $0.4\text{--}1.6 \times 10^6/\text{ml}$  in culture medium II Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated pooled human serum (BioWhittaker, Walkersville, MD),  $2 \times 10^{-5}$  M  $\beta$ -mercaptoethanol (Merck, Darmstadt, Germany), 2 mM sodium-pyruvate (Merck), penicillin (100 U/ml, Gist Brocades, Delft, The Netherlands) and streptomycin (100  $\mu\text{g}/\text{ml}$ , Gibco). The cells were incubated for 30 min at 37° without or with stimulation (phorbol myristate acetate (PMA; Sigma, St. Louis, MO), final concentration 10 ng/ml, and calcium ionophore A23187 (Sigma), final concentration 10  $\mu\text{g}/\text{ml}$ ). Next, the cells were collected by centrifugation and resuspended in washing medium at  $0.4\text{--}1.6 \times 10^6/\text{ml}$ . The cells were irradiated by 3000 rad of  $\gamma$ -irradiation at 0°, centrifuged once more and resuspended in culture medium II at  $0.4\text{--}1.6 \times 10^6/\text{ml}$  and incubated for 24 hr. Supernatants were removed after centrifugation of the cells and were stored at -20°. For some experiments, supernatants were divided into fractions enriched for <30 000 MW molecules and >30 000 MW molecules, respectively. To achieve this, samples were fractionated on centriprep tubes (Amicon, Beverly MA) to obtain a >30 000 MW fraction that was 0.05 times its original volume. This fraction was restored to its original volume with culture medium and subsequently used in experiments. All cultures were at 37° in humidified air containing 5% CO<sub>2</sub>.

### 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 of buffy coats

from healthy subjects. Purified CD8<sup>+</sup> T-cell populations were obtained by negative selection using saturating amounts of anti-CD4, anti-CD16, anti-CD19 and anti-CD56 monoclonal antibodies (mAb; all from CLB Sanquin Blood Supply Foundation, Amsterdam, the Netherlands) at 4° for 30 min. After washing away excess mAb, cells were removed with sheep anti-mouse IgG-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway). The remaining negatively selected cells were subjected once more to the same procedure. The negatively selected cell population consisted of 15% erythrocytes and >84% CD8<sup>+</sup> T cells. CD4<sup>+</sup> T cells, B cells, natural killer (NK) cells, monocytes and polymorphonuclear cells were each below 0.2% as determined by flow cytometry analysis. The CD8<sup>+</sup> T cells were immediately used or kept in culture in medium III (culture medium II plus irradiated PBMC as feeder cells, IL-2 (20 U/ml, Lymfocult, Biotest, Dreiech, Germany) and phytohaemagglutinin (PHA, Murex Diagnostics Ltd, Dartford, UK)). Cell cultures were incubated at 37° in humidified air containing 5% CO<sub>2</sub>.

Polyclonal CD45RA<sup>+</sup> and CD45R0<sup>+</sup> cells were obtained from a freshly purified polyclonal CD8<sup>+</sup> T-cell population. Cells were divided into two portions and incubated at 4° for 30 min with saturating amounts of anti-CD45RA-fluorescein isothiocyanate (FITC) or anti-CD45R0 (Beckton Dickinson (BD), San José, CA), respectively. The latter were subsequently incubated with goat anti-mouse F(ab)<sub>2</sub>-FITC (CLB) for 30 min at 4°. Cells were then sorted by fluorescence-activated cell sorting (FACS Star Plus (BD)). Experiments were performed with the negatively selected fractions: CD45R0<sup>-</sup> (=CD45RA<sup>+</sup> naive cells) and the CD45RA<sup>-</sup> (=CD45R0<sup>+</sup> memory cells). The cells were used in the assays immediately.

T-lymphocyte clones were prepared as described.<sup>8,20</sup> 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. For the experiments described in this study, we selected clones with a Th0 phenotype, producing both IFN- $\gamma$  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  $0.4\text{--}0.8 \times 10^6$  cells/ml at 37°. 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  $0.4 \times 10^6/\text{ml}$  in medium II.

### T-cell proliferation and cytokine production

Roundbottom plates (96-well, Costar, Cambridge, MA) were coated in quintuplicate overnight with anti-CD3 mAb, after which the plates were washed three times with PBS. T-cells (40 000 cells/well) were added, and 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. For

assay of cytokine production, the plates were centrifuged, supernatants of five parallel wells were pooled and stored at  $-20^{\circ}$  until the day of assay. T-cell proliferation was assessed in parallel by measuring [ $^3$ H]thymidine incorporation. 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- $\gamma$ , IL-5 and IL-6 contents were measured by enzyme-linked immunosorbent assay (ELISA).<sup>15,21,22</sup> Briefly, plates coated with mAb anti-IFN- $\gamma$  (MD2), anti-IL-5 (TRFK-5) or anti-IL-6 (mIL-6-16 M) were incubated with sample together with an excess of biotinylated mAb anti-IFN- $\gamma$  (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- $\gamma$ /ml, 47 pg IL-5/ml and 3 pg IL-6/ml. Levels of IL-4, IL-8, PGE<sub>2</sub>, TxB2 were assessed using commercially available kits (CLB, CLB, Amersham, Amersham, UK and Amersham, respectively). Lower limits of detection were 1.95 pg IL-4/ml, 1.02 pg IL-8/ml, 10 pg prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)/ml, and 3.6 pg TxB2/ml.

#### Reverse transcription-polymerase chain reaction (RT-PCR) for IFN- $\gamma$

Polyclonal CD8<sup>+</sup> T-cells ( $1 \times 10^6$  per well) were cultured for 0 min, 30 min, 1 hr, 2 hr and 4 hr without or with (stimulated) mast cells in the presence of anti-CD3. Total RNA was isolated using TRIzol Reagent (Gibco). First strand cDNA was synthesized from total RNA using Superscript II RNase H-RT (Gibco). PCR amplification was carried out in PCR buffer (Eurogentec, Maestricht, The Netherlands; final volume 50  $\mu$ l) containing MgCl<sub>2</sub> (Eurogentec), dNTP (Pharmacia, Uppsala, Sweden), Taq DNA polymerase (Goldstar, Eurogentec) and  $\beta$ 2 microglobulin- or IFN- $\gamma$ -specific primers (Eurogentec). We coamplified PQA1, a multispecific internal control.<sup>23</sup> The mixture was covered by mineral oil (Sigma) and denatured at 95 $^{\circ}$  for 5 min. Optimal conditions for the PCR reaction were 35 cycles (94 $^{\circ}$ , 60 $^{\circ}$  and 72 $^{\circ}$ , each for 1 min).<sup>24</sup> 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 dilutions of samples were used in which intensity of the bands of the T-cell product differed not more than factor 2 from the intensity of the PQA1 product. Absolute amounts of IFN- $\gamma$  mRNA were calculated using the known amount of the PQA1 product and were related to the absolute amounts of  $\beta$ 2-microglobulin which were calculated in the same way as IFN- $\gamma$ .

#### 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

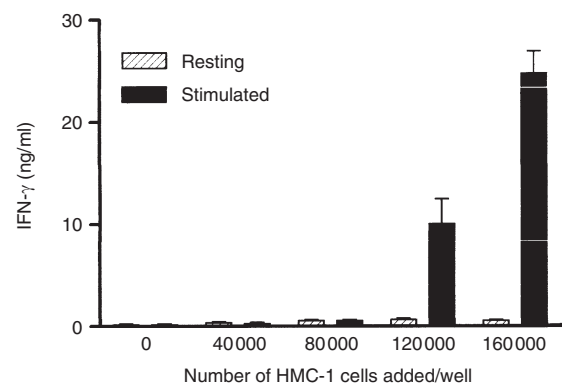
### Products from HMC-1 cells

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,  $49 \pm 26$  pg/ml and  $1020 \pm 116$  pg/ml (mean  $\pm$  standard error of the mean, sem;  $n=5$ ), respectively; PGE<sub>2</sub>,  $208 \pm 57$  and  $278 \pm 57$  pg/ml ( $n=2$ ), respectively; IL-6, 10 and 32 pg/ml, respectively; leukotriene B<sub>4</sub>, 223 and 541 pg/ml, respectively; and TxB2, 611 and 855 pg/ml, respectively. IFN- $\gamma$ , IL-4, IL-5 or IL-12 could not be detected in the supernatants.

### Effects of HMC-1 cells on polyclonal CD8<sup>+</sup> T cells and polyclonal CD4<sup>+</sup> T cells

Resting HMC-1 cells enhanced the proliferation of anti-CD3 stimulated polyclonal CD8<sup>+</sup> T cells  $1.5 \pm 0.2$  fold (mean  $\pm$  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  $38 \pm 7\%$  of control (mean  $\pm$  SEM,  $n=8$  experiments). The irradiated HMC-1 cells and PBMC alone showed very low [ $^3$ H]thymidine incorporation. HMC-1 mast cell-line cells had essentially the same effects on polyclonal CD4<sup>+</sup> cells.

Figure 1 shows an experiment with polyclonal CD8<sup>+</sup> T cells. The cells (40 000 cells/well) were stimulated with anti-CD3 and incubated together with increasing numbers of mast cells. Without mast cells added,  $200 \pm 41$  pg (mean  $\pm$  SEM) IFN- $\gamma$ /ml was measured in the supernatant.



**Figure 1.** IFN- $\gamma$  production at 24 hr by anti-CD3 stimulated polyclonal CD8<sup>+</sup> T lymphocytes (mean  $\pm$  1 standard deviation). CD8 T cells, 40 000 cells per well. Where indicated resting HMC-1 cells or stimulated HMC-1 cells ranging from 40 000 to 160 000 cells per well were added.

The addition of resting HMC-1 cells up to 160 000 cells per well enhanced IFN- $\gamma$  production to  $700 \pm 70$  pg/ml. Stimulated mast cells increased IFN- $\gamma$  production up to  $10.1 \pm 0.7$  and  $24.7 \pm 0.8$  ng/ml, when 80 000 and 160 000 cells per well were added, respectively. The results from 6 experiments with polyclonal CD4<sup>+</sup> T-cell populations and 13 experiments with polyclonal CD8<sup>+</sup> T-cell populations are shown in Table 1. For CD4<sup>+</sup> T cells the ratio (IFN- $\gamma$  production in the presence of anti-CD3 plus stimulated mast cells)/(IFN- $\gamma$  production in the presence of anti-CD3) was  $59.2 \pm 6.8$  (mean  $\pm$  SEM) and for CD8<sup>+</sup> T cells this ratio was  $35.1 \pm 17.1$ . The increase of IFN- $\gamma$  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 in parallel experiments. 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 PMBC. The stimulated PBMC induced a similar increase of IFN- $\gamma$  production as resting PBMC (Table 1). Thus, the effects of stimulated PBMC were qualitatively and quantitatively different from those of stimulated HMC-1 cells.

Next, we measured the effect of mast cells on IFN- $\gamma$  mRNA. Polyclonal CD8<sup>+</sup> T cells were cultured up to 4 h without or with (stimulated) mast cells in the presence of anti-CD3. Table 2 shows that the activated mast cells

increased IFN- $\gamma$  mRNA already at 30 min. IFN- $\gamma$  mRNA increased up to at least 4 h of culture, and resting mast cells had only a very small effect. IFN- $\gamma$  protein in the supernatants from anti-CD3 stimulation and from stimulation with resting mast cells was below 48 pg/ml. Stimulated mast cells enhanced the IFN- $\gamma$  production to 88 pg/ml and 1 ng/ml at 2 hr and 4 hr, respectively. The results of these experiments showed that stimulated mast cells induced a rapid increase of IFN- $\gamma$  mRNA. When CD8 T cells were omitted and only unstimulated or stimulated mast cells were incubated no significant amounts of IFN- $\gamma$  mRNA were detected.

The production of IL-4 by the polyclonal CD8<sup>+</sup> 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<sup>+</sup> T cells. Neither the irradiated mast cells nor the irradiated PBMC produced detectable levels of IFN- $\gamma$ , IL-4 or IL-5.

#### CD8<sup>+</sup> CD45RA<sup>+</sup> and CD8<sup>+</sup> CD45R0<sup>+</sup> cells

CD45R0<sup>+</sup> T cells have a higher IFN- $\gamma$  production capacity than the CD45RA<sup>+</sup> T cells.<sup>25</sup> To test whether the increase of IFN- $\gamma$  production might be related to a differentiation of T cells the effect of stimulated mast cells was studied with purified CD8CD45RA<sup>+</sup> and CD8CD45R0<sup>+</sup> T cells. Anti-CD3 stimulated naive cells did not produce detectable amounts of IFN- $\gamma$ . Stimulated (but not resting) mast cells induced the naive cells to produce 550 pg IFN- $\gamma$ /ml. Anti-CD3 stimulated memory cells produced 393 pg IFN- $\gamma$ /ml. Resting and stimulated mast cells led to 4.4-fold and 12.2-fold increased IFN- $\gamma$  production, respectively. Thus, the effects exerted by HMC-1 cells were similar for both naive and memory CD8<sup>+</sup> T-cells. When stimulated or unstimulated mast cells were incubated without CD45R0<sup>+</sup> or CD45RA<sup>+</sup> cells IFN- $\gamma$  in the supernatants was below the limit of detection of the ELISA.

**Table 1.** IFN- $\gamma$  production at 24 h by anti-CD3 stimulated polyclonal CD4<sup>+</sup> and polyclonal CD8<sup>+</sup> T lymphocytes

	CD4	CD8
<b>Stimulus</b>		
Anti-CD3 alone	0.61 $\pm$ 0.7*	0.61 $\pm$ 0.20
+Resting HMC-1	1.31 $\pm$ 1.4	2.13 $\pm$ 0.12
+Stimulated HMC-1	15.2 $\pm$ 13.3	14.7 $\pm$ 4.8
+Resting PBMC	1.0 $\pm$ 0.8	1.14 $\pm$ 0.14
+Stimulated PBMC	not done	1.08 $\pm$ 0.18
<b>Ratios</b>		
Resting HMC-1/anti-CD3	2.55 $\pm$ 0.9*	4.23 $\pm$ 1.1
Stimulated HMC-1/anti-CD3	59.2 $\pm$ 6.8†	35.1 $\pm$ 17.1†
Resting PBMC/anti-CD3	2.35 $\pm$ 1.0	1.65 $\pm$ 0.39‡
Stimulated PBMC/anti-CD3	not done	1.70 $\pm$ 0.28

Where indicated resting HMC-1 cells, stimulated HMC-1 cells or resting PBMC were added to the wells.

CD4 cells, six different experiments with cells from one donor. CD8 cells, 13 different experiments with cells from three donors, except for stimulated PBMC for which four different experiments are presented.

\*ng IFN- $\gamma$ /ml, mean  $\pm$  SEM.

†higher than the ratio resting HMC-1/anti-CD3,  $P < 0.001$ ; higher than the ratio resting PBMC/anti-CD3,  $P < 0.01$ ; higher than the ratio stimulated PBMC/anti-CD3,  $P < 0.01$ .

‡Lower than resting HMC-1/anti-CD3,  $P < 0.02$ .

**Table 2.** Quantities of IFN- $\gamma$  mRNA (in fg/100 fg  $\beta$ 2 microglobulin mRNA) present in the cultures of anti-CD3 stimulated polyclonal CD8<sup>+</sup> T lymphocytes ( $1 \times 10^6$  cells) without or with (stimulated) HMC-1 cells ( $4 \times 10^6$  cells)

Time (hr)	Polyclonal CD8 <sup>+</sup> T-cells	+Resting HMC-1 cells	+Stimulated 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

ND, not done.

Resting and stimulated mast cells incubated for the various times without CD8 T cells showed less than 0.07 fg IFN- $\gamma$  mRNA per 100 fg  $\beta$ 2 microglobulin mRNA.



### Effect of mast cell supernatant and individual products

Mast cells were stimulated as described in methods and incubated for 24 hr at 37°. Supernatant was collected and added in a 1:2 or 1:4 dilution to polyclonal CD8<sup>+</sup> T cells. The 1:2 diluted supernatant decreased proliferation from 11 000 c.p.m. to 6000. When the supernatant was diluted to 1:4 the proliferation was as with anti-CD3 alone. The IFN- $\gamma$  production was  $1.7 \pm 1.1$  (mean  $\pm$  SD,  $n=3$ ),  $9.1 \pm 4.2$  and  $1.0 \pm 1.1$  ng/ml for anti-CD3 stimulated cells without supernatant, with 1:2 diluted supernatant and 1:4 diluted supernatant added, respectively, the condition with the 1:2 dilution being significantly higher than the others ( $P < 0.05$ ). Supernatants from not-stimulated mast cells and supernatants from irradiated PBMC that had been stimulated like the mast cells had no significant effects.

The following mediators and cytokines were incubated with the CD8<sup>+</sup> T cells, separately at various doses during 24 hr to investigate whether they were able to mimic the effect of stimulated mast cells or mast cell supernatant. Heparin (22 ng/ml to 225  $\mu$ g/ml), histamine ( $10^{-8}$ – $10^{-4}$  M), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ; 10 pg/ml to 10 ng/ml), IL-1 $\beta$  (10 pg/ml to 10 ng/ml), IL-8 (5–500 pg/ml), PGD2 ( $10^{-8}$ – $10^{-6}$  M), PGE<sub>2</sub> ( $10^{-8}$ – $10^{-6}$  M), IL-4 (1 pg/ml to 10 ng/ml) (all potential products from mast cells<sup>17,26</sup>), IL-12 (5–500 pg/ml) and IL-18 (0.5–8 nM) (known stimulators of IFN- $\gamma$  production<sup>27</sup>). Though some of them had a small stimulatory effect on IFN- $\gamma$  production, none of the mediators could account for the increase of IFN- $\gamma$  production we found when T cells were cultured with the stimulated HMC-1 cells or supernatant from stimulated mast cells.

Next, experiments were performed in which mast cell supernatants and medium were divided into fractions enriched for <30 000 MW molecules and >30 000 MW molecules, respectively. These enriched fractions were added to polyclonal CD8<sup>+</sup> T cells and IFN- $\gamma$  production was measured. Upon anti-CD3 stimulation polyclonal CD8<sup>+</sup> cells produced 680 pg/ml IFN- $\gamma$ . The >30 000 MW enriched fraction of the supernatant of stimulated mast cells increased IFN- $\gamma$  production 23 times, whereas the low molecular weight fraction had no effect.

### Different kinds of T-cell stimuli

Polyclonal CD8<sup>+</sup> 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- $\gamma$  and supernatant of resting mast cells had no effect (Table 3). Supernatant of stimulated mast cells led to an induction of IFN- $\gamma$  production of 6.2 ng/ml. Similar results were seen for the stimuli PHA, PMA and Con A. Soluble anti-CD3<sup>+</sup>, soluble anti-CD28, and ionomycin induced low levels of IFN- $\gamma$  production (0.3 ng/ml and 0.5 ng/ml, respectively). Then, the supernatant of stimulated mast cells increased IFN- $\gamma$  production to 12.5 ng/ml and 4.7 ng/ml, respectively, and supernatant of resting mast cells hardly affected IFN- $\gamma$  production. When T cells were

stimulated by PMA + ionomycin, there was already a high production of IFN- $\gamma$  (13.8 ng/ml). Here, resting mast cells had no effect and the supernatant of stimulated mast cells inhibited IFN- $\gamma$  production to 34% of control.

### Effects of HMC-1 cells on CD4<sup>+</sup> and CD8<sup>+</sup> T-cell clones

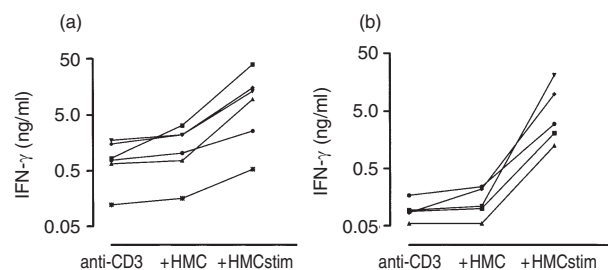
The effect of HMC-1 cells was tested on homogeneous and further defined cell populations, CD8<sup>+</sup> T-cell clones and CD4<sup>+</sup> T-cell clones obtained from healthy and allergic subjects (Figs 2–4). The anti-CD3 induced production of IFN- $\gamma$  by CD8<sup>+</sup> T-cell clones was higher than that by CD4<sup>+</sup> T-cell clones (mean ng/ml  $\pm$  SEM:  $0.95 \pm 0.25$  and  $0.1 \pm 0.02$ , respectively) ( $P < 0.01$ , MWU test). Resting mast cells led to no or small increases of IFN- $\gamma$  production (Fig. 2). Stimulated HMC-1 cells caused additional increases of IFN- $\gamma$  production for all clones studied, resulting in similar high production by CD8<sup>+</sup> and CD4<sup>+</sup> T-cell clones.

IL-4 production was mostly decreased after addition of resting mast cells (Fig. 3a,b). In most of the clones IL-4

**Table 3.** IFN- $\gamma$  production (ng/ml) by polyclonal CD8<sup>+</sup> T cells

Stimuli	Additions		
	None	Supernatant of resting mast cells	Supernatant of stimulated mast cells
None	<0.06	<0.06	6.2
Anti-CD3 + anti-CD28	0.28	0.36	12.5
PHA (0.5 $\mu$ g/ml)	<0.06	<0.06	5.2
PMA (1 ng/ml)	<0.06	<0.06	12.5
Ionomycin (1 $\mu$ g/ml)	0.51	0.39	2.3
PMA (1 ng/ml) + ionomycin (1 $\mu$ g/ml)	13.8	13.0	4.7
Con A (0.5 $\mu$ g/ml)	<0.06	0.09	7.3

Cells were incubated at 40 000 cells/well in quintuplicate in medium, or in supernatant from stimulated or resting mast cells together with different T-cell stimuli. Culture supernatants were harvested after 24 hr.



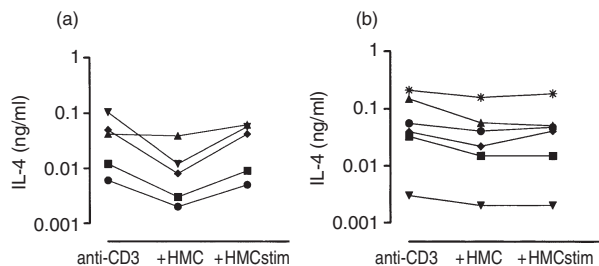
**Figure 2.** (a) IFN- $\gamma$  production at 24 hr by anti-CD3 stimulated clonal CD8<sup>+</sup> T lymphocytes. Clones were obtained from the peripheral blood of three healthy control subjects. Where indicated resting HMC-1 cells or stimulated HMC-1 cells were added to the wells. (b) As (a) but clonal CD4<sup>+</sup> T lymphocytes from three healthy control subjects.

production was not affected by stimulated mast cells. Several T-cell clones produced IL-5. The production of IL-5 by CD8<sup>+</sup> T-cell clones from healthy subjects was not affected by resting mast cells (Fig. 4). In some of the CD4<sup>+</sup> T-cell clones the stimulated mast cells enhanced the IL-5 production slightly.

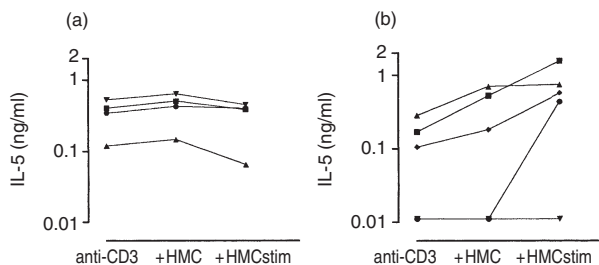
Supernatants from unstimulated or stimulated mast cells had similar effects on T-cell clones with respect to proliferation and cytokine production as observed for their effects on polyclonal T cells.

### IL-4 recovery

Because mast cells were found to be able to degrade<sup>28</sup> 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 hr was 80 and 58%, respectively, of the control situation where IL-4 was incubated without mast cells. Thus, there



**Figure 3.** (a) IL-4 production at 24 hr by anti-CD3 stimulated clonal CD8<sup>+</sup> T lymphocytes. Clones were obtained from the peripheral blood of three allergic asthmatic subjects. Where indicated resting HMC-1 cells or stimulated HMC-1 cells were added to the wells. (b) As (a) but clonal CD4<sup>+</sup> T lymphocytes from 1 allergic asthmatic subject.



**Figure 4.** (a) IL-5 production at 24 hr by anti-CD3 stimulated clonal CD8<sup>+</sup> T lymphocytes. Clones were obtained from the peripheral blood of three healthy subjects. Where indicated resting HMC-1 cells or stimulated HMC-1 cells were added to the wells. (b) As (a) but clonal CD4<sup>+</sup> T lymphocytes.

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.<sup>19,29</sup> Although the cell-line cells have been shown to share many characteristics with native mast cells<sup>18</sup> they lack the  $\gamma$ -chain of the Fc $\epsilon$ I 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- $\gamma$  mRNA and IFN- $\gamma$  protein production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, both in polyclonal T-cell populations and in T-cell clones. The increase of the IFN- $\gamma$  production was already detectable at 2 hr 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.*<sup>30</sup> In contrast, Huels *et al.*<sup>31</sup> found that coculturing of anti-dinitrophenol-IgE sensitized bone-marrow-derived mast cells with naive spleen CD4<sup>+</sup> T cells led to a decrease of IFN- $\gamma$  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 cocultures. When we added IL-4 to the polyclonal CD8<sup>+</sup> T-cells we also found a (dose-dependent) decrease of IFN- $\gamma$  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- $\gamma$  production. We have tested other possible HMC-1 cell products like histamine, PGE<sub>2</sub>, PGD<sub>2</sub>, TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and IL-8, and they did not induce large stimulation of IFN- $\gamma$  production when added separately. Furthermore, we have found that the IFN- $\gamma$  enhancing cytokines IL-12 and IL-18 could not account for the increase of IFN- $\gamma$  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- $\gamma$  production was obtained by experiments in which we separated culture medium and (resting and stimulated) mast-cell supernatants into fractions enriched for either <30 000 MW molecules or >30 000 MW molecules. The IFN- $\gamma$  enhancing capacity was exclusively found in the >30 000 MW fraction of the stimulated mast-cell supernatant. We were not able

to identify the components which are responsible for the increase in IFN- $\gamma$  by the T cells.

Recent studies in mice have demonstrated that mast cells were able to enhance T-cell proliferation and IFN- $\gamma$  production by T cells both in *in-vivo* and in *in-vitro* systems.<sup>30,32,33</sup> This effect was mediated by exosomes secreted by mast cells, which are small particles containing a variety of biologically active proteins.<sup>32</sup> It was not clear, however, which of these molecules caused the effect.<sup>32</sup> Furthermore, it appeared that other cells than T cells were required in addition to obtain the full enhancement of IFN- $\gamma$  production.<sup>30</sup> In contrast, in this study the mast-cell derived factor(s) inhibited T-cell proliferation in most conditions, and no other cells were required for the effect on IFN- $\gamma$  production. We have not studied whether exosomes are produced by the mast cell-line cells applied in our study. The factor(s) involved in this study with human cells resisted at least one freeze-thaw cycle, and were bigger than 30 000 MW in size.

Because human mast cells were found to release IL-4 protein,<sup>34</sup> they are commonly thought to favour differentiation of T cells to the Th2 phenotype.<sup>35-37</sup> However, a study with human lung mast cells (obtained from normal lung) failed to detect release of IL-4 protein<sup>38</sup> 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.<sup>39</sup> 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.<sup>16</sup> IL-4 mRNA was higher in bronchial mucosal tissue from allergic asthma patients than from non-atopic controls,<sup>5</sup> and part of the IL-4 protein was associated with mast cells.<sup>6,35</sup> 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 vs. 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.

IFN- $\gamma$  may act as a proinflammatory cytokine.<sup>40</sup> It may be hypothesized 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- $\gamma$  by local T lymphocytes. In fact, high levels of IFN- $\gamma$ <sup>10</sup> and an increase of IFN- $\gamma$ -positive T cells<sup>41</sup> have been demonstrated in bronchoalveolar lavage fluid shortly after local allergen exposure.

Furthermore, IFN- $\gamma$  is well-recognized for its inhibitory activity on the differentiation towards Th2 cells.<sup>42</sup> Some studies in mice and rats indicate a role for CD8<sup>+</sup> T cells in the *in vivo* down-regulation of IgE production by the production of IFN- $\gamma$ .<sup>43</sup> A similar mechanism may be operative in human cells, though the reactions involved may be more complicated than just IFN- $\gamma$  production.<sup>43</sup> Thus, a long-lasting or a chronic effect of an increased IFN- $\gamma$  expression

under the influence of mast cell activation may be the induction of a relative skewing to Th1 reactions, whereas an acute effect of IFN- $\gamma$  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- $\gamma$  over IL-4 could account for a negative feedback system by which mast cells locally down-regulate allergen-induced Th2 responses both via the CD8<sup>+</sup><sup>44</sup> and the CD4<sup>+</sup> T cells. 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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