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Up-regulation of mucin secretion in HT29-MTX cells by the pro-inflammatory cytokines tumor necrosis factor-a and interleukin-6.

Abstract

The pro-inflammatory cytokines IL-6 and TNF-a have been implicated in the pathogenesis of otitis media with effusion (OME). A disease where goblet cells proliferate in a modified respiratory epithelium, leading to the accumulation of a mucin-rich effusion in the middle ear cleft. The MUC5AC and MUC5B mucin gene products have been identified as components of these effusions. To determine

the effect of IL-6 and TNF-a on MUC5AC and MUC5B secretion we have used HT29-MTX goblet cells, which secrete both types of mucins. MUC5AC and MUC5B mucin secretion was measured by an enzyme-linked immunosorbent assay (ELISA) using a specific monoclonal antibody NCL-HGM-45M1 and polyclonal antiserum TEPA, respectively. Time response (0-72 hours) and dose response (1.5-150 ng/ml) studies were carried out. IL-6 and TNF-a stimulated MUC5AC and MUC5B mucin secretion in a time dependent manner, both in pre-confluent and post-confluent cells. IL-6 (15 ng/ml and 20 ng/ml) produced a low and prolonged stimulation of mucin secretion that persisted for 72 hours, with peak response at 24 hours after induction. The IL-6-mediated mucin secretion at 24 hours was concentration-dependent, with a maximal effect at 15 ng/ml. TNF-a (20 ng/ml) induced rapid stimulation of mucin secretion within the first 24 hours, with peak response at 7 hours after induction. IL-6 and TNF-a exposure significantly increased MUC5AC secretion, but not MUC5B secretion. Maximal levels of cytokine-induced mucin secretion were detected in pre-confluent cells that showed one and a half- and two-fold increases in MUC5AC secretion after IL-6 and TNF-a stimulation, respectively, in comparison with post-confluent cells. The results presented here suggest that IL-6 and TNF-a generate a differential up-regulation of mucin secretion and thus contribute to the expression of mucin genes in inflammatory responses.

Full text

INTRODUCTION

IL-6 and TNF-alpha are multifunctional cytokines involved in the regulation of the immune response and inflammation, and have been found to regulate the local and systematic reactions accompanying different types of inflammation [1-3].

Otitis media with effusion (OME) is a disease characterized by an increase in goblet cells and submucosal glands in middle ear cleft mucosa. These cellular changes are accompanied by the accumulation of viscous effusions, the main component of which is mucin. Investigations by our group and others [4-6] have demonstrated a high level of IL-6 (up to 173.9 pg/mg of total protein) [6] and TNF-alpha (up to 67 pg/mg of total protein) [4] in effusions. These high levels of cytokines, together with the hypersecretion of mucin, allowed us to propose the potential involvement of pro-inflammatory cytokines TNF-alpha and IL-6 in the

regulation of mucin gene expression and mucus secretion during the local inflammatory process in the middle ear, leading to the development of OME. Studies on the antigenic identity of middle ear mucin [7] have identified epitopes for two mucins (MUC5AC and MUC5B) in middle ear effusions. Our preliminary investigations of the influence of TNF-alpha on mucin secretion have shown that TNF-alpha up-regulates MUC5AC secretion [8].

The present study was designed to determine the role of TNF-alpha and IL-6 in MUC5AC and MUC5B secretion and to analyze the dynamics between pro-inflammatory cytokine induction and MUC5AC and MUC5B mucin secretion with the *in vitro* model, mucin-secreting HT29-MTX cell line.

MATERIALS AND METHODS

Materials

The human colon carcinoma-derived, mucus-secreting HT29-MTX cell line was kindly provided by Dr. Thecla Lesuffleur (INSERM U. 178, France).

Human gastric mucin, mouse monoclonal antibody (NCL-HGM-45M1), MUC5AC gene product-specific were purchased from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK).

Purified mucin from thick effusions of anatomically normal children was used to prepare polyclonal antiserum (TEPA) in rabbits. The polyclonal antibody TEPA reacts strongly with high molecular weight mucin from human saliva, which was identified as MUC5B [9]. Purified mucin from human saliva was used for the titration of the TEPA antibody. NCL-HGM-45M1 and TEPA were used in this study at dilutions of 1:50 and 1:500, respectively. The antibodies produced similar reactivities against their respective standards (on a wt/wt basis).

The specificity of the TEPA polyclonal antiserum was determined by immunohistochemical and papain digestion studies. The pattern of staining with the TEPA antibody [10] showed negative results in stomach and ileum tissues, positive results in salivary glands and tracheal submucosal glands, which have been previously reported to express the MUC5B gene [11], and strong staining of the mucous glands of the middle ear mucosa. There was no staining in cells and tissues, histologically identified as non-mucus secreting. Papain digestion of

thick effusion mucin, which removes the non/sparsely glycosylated regions, abolished TEPA reactivity and demonstrated that TEPA antiserum was not recognising the major carbohydrate-containing domain or any carbohydrate epitopes on that domain. TEPA almost certainly recognises a protein epitope in the non-tandem repeat region [7].

Human recombinant TNF-alpha, human recombinant IL-6, cell culture flasks (25 mm^2), collagen PRO COAT 24-well plates, Dulbecco's modified Eagles minimum essential medium (DMEM); fetal bovine serum (FBS); Dulbecco's phosphate-buffered saline (DPBS); trypsin-EDTA solution (1X); bovine serum albumin fraction V, (BSA); anti-mouse IgG (whole molecule) peroxidase conjugated; anti-rabbit IgG (whole molecule) peroxidase conjugated; 3,3'-diaminobenzidine tetrahydrochloride (DAB) liquid substrate system, polyoxyethylenesorbitan monolaurate (Tween 20), Trypan blue solution (0.4%) were purchased from Sigma Chemical Co. Nitrocellulose transfer membrane 0.45 mm (Protran) and blotting paper (GB002) for immunoblotting were obtained from Schleicher@Schuell Company (Germany).

METHODS

Cell culture

HT29-MTX cells were grown in 25 cm^2 plastic T-flasks in DMEM medium supplemented with 10% heat-inactivated FBS at 37° C in a 10% CO_2/90% air atmosphere. For maintenance, cells were seeded at 3 x 10^6 cells in 25 cm^2 flasks in 10 ml medium. The medium was changed every second day. Cells were passaged every 15 days at the stage of ~ 80% of confluence, using trypsin 0.0125% in 0.53 mM EDTA in Ca^{2+} Mg^{2+}-free DPBS. The cell viability in cytokine experiments was checked by trypan blue exclusion.

Exposure to IL-6 - time-response study

HT29-MTX cells were seeded (3 x 10^4 cells/well) in 24-well plates (2 ml of medium) and grown for 7 days for the pre-confluent stage or 17 days for the post-confluent stage, in DMEM + 10% FBS medium. Before exposure to IL-6, cells were pre-incubated for 16 hours in serum-free medium. After exposure to IL-6 (20 ng/ml and 15 ng/ml) cells were grown in serum-free medium for 3 days. The medium was not changed during this time. In order to determine the time-

dependent mucin release, aliquots of the medium, containing secreted mucin, were taken within the 3 days, every 2 hours, 6 times per day (started 2 hours after initial IL-6 addition).

Exposure to IL-6 - dose-response study

In dose response experiments with IL-6, HT29-MTX cells were seeded and grown for 7 days (pre-confluent stage) as described previously. On day 8 the cells were exposed to different concentrations of IL-6 (1.5 ng/ml, 2 ng/ml, 5 ng/ml, 15 ng/ml, 20 ng/ml, 50 ng/ml, 150 ng/ml) in serum-free medium. Aliquots of media with released mucin were taken after 24 hours of incubation and subjected to mucin immunoassay.

*Exposure to TNF-*alpha *and IL-6 - time-response study*

HT29-MTX cells were seeded and grown until the pre-confluent stage as described above. On day 7, cells were treated in parallel with either IL-6 (15 ng/ml) or TNF-alpha (20 ng/ml), and grown in FBS-free medium for another 3 days. To trace differences in IL-6- and TNF-alpha-induced mucin release, aliquots of medium were taken every hour between 2 and 12 hours after cytokine addition, as it has been shown [8], that TNF-alpha induces MUC5AC secretion from HT29-MTX cells within the first 12 hours after induction. Aliquots of medium were then taken every 12 hours up to 72 hours.

Immunoassay of MUC5AC and MUC5B mucin

MUC5AC and MUC5B mucin secretion from HT29-MTX cells was measured by an indirect ELISA method with slot-blotting technique, and using monoclonal antibody NCL-HGM-45M1 and polyclonal antiserum TEPA, respectively, for MUC5AC and MUC5B identification.

Aliquots of cell culture media (100 mul) were blotted onto nitrocellulose sheets (0.45 mum) and incubated overnight at 4° C in PBS with 2% (w/v) BSA. Nitrocellulose sheets were incubated for 2 hours at room temperature with NCL-HGM-45M1 and TEPA used at dilutions of 1:50 and 1:500, respectively, in PBS containing 1% BSA. After 2 hours, sheets were washed twice with PBS, containing 0.5% Tween 20, and 3 times with PBS, and incubated for another hour at room temperature with anti-mouse IgG and anti-rabbit IgG peroxidase

conjugates (1:10,000) for NCL-HGM-45M1 and TEPA, respectively, in PBS containing 1% BSA. After one hour, the sheets were washed as described above. The colour reaction was developed with 3'3'-diaminobenzidine tetrahydrochloride in peroxide solution. Staining of the slots was quantitated by a Shimadzu CS 930 dual wavelength scanning densitometer at 595 nm.

Statistical analyses

Every time point in the cytokine experiment had its own experimental well and control well with unstimulated HT29-MTX cells. Mucin secretion by HT29-MTX cells after cytokine stimulation was measured against the control. Every time point was repeated in triplicate.

Results were calculated by dividing the relative intensity reading in arbitrary units (AU), corresponding to mucin secreted during experimental time points after cytokine stimulation, by the relative intensity reading (AU), corresponding to mucin secreted by unstimulated HT29-MTX cells in the control well. Because HT29-MTX cells mucin output varied from experiment to experiment, the results were expressed as a percentage of the control to allow inter-experiment comparison.

Control and cytokine-induced mucin secretion were compared *via* an unpaired Student's t-test. An one-way ANOVA was used to determine statistically significant differences between study groups and controls. Values with $P < 0.05$ were considered significant.

RESULTS

Effects of IL-6 on mucin secretion

The study of IL-6-induced mucin secretion in the HT29-MTX cell line showed an up-regulation of MUC5AC and MUC5B secretion by IL-6 (20 ng/ml) both in pre- and post-confluent cells. IL-6-induced mucin secretion in HT29-MTX cells was initially detected at 4 hours (3% above the control for MUC5AC), and persisted for up to 72 hours (Figure 1). Mucin secretion peaked after 24 hours of incubation both for MUC5AC and MUC5B in the pre- and post-confluent cells.

IL-6-mediated MUC5AC secretion increased slowly during the first 12 hours (13% higher than the control) of incubation, increased to a maximum level at 24 hours and was 30% higher, and significantly different from the control for pre-confluent cells (Figure 1A), and 21% higher for post-confluent cells (Figure 1B). The level of MUC5AC secretion then fell slowly during the next 48 hours and returned to the control level at 72 hours. However, MUC5AC secretion was still significantly higher than the control between 26 and 34 hours of incubation, 27 and 21%, respectively, for pre-confluent cells (Figure 1A).

IL-6 stimulated MUC5B secretion in the same time-dependent manner as MUC5AC, but the level of IL-6-induced MUC5B secretion was 3 times lower in comparison with MUC5AC, and secretion at 24 hours was only 10% higher than the control for pre-confluent cells (Figure 1A) and 7% higher for post-confluent cells (Figure 1B).

Exposure to IL-6: dose-response study

Exposure of pre-confluent cells to different concentrations of IL-6 (Figure 2) demonstrated that IL-6-mediated mucin secretion in HT29-MTX cells at 24 hours was concentration-dependent, and IL-6 significantly increased MUC5AC secretion with peak responses at concentrations of 15 ng/ml and 20 ng/ml (33 and 30%, respectively, above the control). IL-6 increased MUC5B secretion also in a concentration-dependent manner, but the peak response at an IL-6 concentration of 15 ng/ml was only 9.6% higher than the control. Thus, IL-6 (15 ng/ml) stimulated the maximum level of mucin secretion in HT29-MTX cells both for MUC5AC and MUC5B mucins (Figure 2). IL-6 at a concentration of 15 ng/ml was therefore used in subsequent experiments.

*Effects of TNF-*alpha *on mucin secretion*

Parallel time studies of the cytokine-induced mucin secretion by pre-confluent cells demonstrated different levels of mucin release after exposure to IL-6 (15 ng/ml) and TNF-alpha (20 ng/ml) (Figure 3). TNF-alpha stimulated mucin secretion in a time-dependent manner, both for MUC5AC and MUC5B. An initial increase in TNF-alpha-mediated mucin secretion was detected at 2 hours (11% higher than the control) after exposure and persisted for 48 hours. TNF-alpha-induced MUC5AC secretion started immediately after exposure, increased within the first 5 hours (25% higher than the control) and attained a maximal

level at 7 hours (56% above the control). After 8 hours of incubation, the level of TNF-alpha -induced MUC5AC secretion began fall progressively over the next 16 hours and returned to the initially detected level of secretion at 24 hours (12% higher than the control (Figure 3A).

TNF-alpha stimulated MUC5B secretion in the same manner as MUC5AC, but this stimulation was 3.5 times lower, and secretion at 7 hours was only 15% above controls (Figure 3B).

TNF-alpha-mediated mucin secretion increased sooner, peaked earlier and was shorter in comparison with
IL-6-induced mucin secretion.

Influence of cell growth stage on cytokine-induced mucin secretion

TNF-alpha (20 ng/ml) and IL-6 (15 ng/ml) induced mucin secretion both in pre-confluent and in post-confluent cells (Figure 4). Maximal levels were detected in pre-confluent cells, both for MUC5AC and MUC5B. Cytokine-induced mucin secretion in post-confluent cells was essentially lower.

TNF-alpha provoked hypersecretion of MUC5AC after 7 hours in pre-confluent cells and the levels of this secretion were 53-56% higher compared to the control, whereas the responses to the same stimulation in post-confluent cells were only 24-26% higher than the control. For IL-6-induced MUC5AC secretion, the influence of the cell's growth stage was not as great. However, IL-6-induced MUC5AC secretion in pre-confluent cells (28-30% above the control) was 1.5 times higher than in post-confluent cells (18-20% above the control).

TNF-alpha- and IL-6-induced MUC5B secretion in HT29-MTX cells did not change significantly with the duration of cell culture, but the level of this secretion in pre-confluent cells was higher compared to post-confluent cells (Figure 4).

In general, the stage of cell growth was important for cytokine-induced MUC5AC secretion but not for cytokine-induced MUC5B secretion.

DISCUSSION

Excessive mucus secretion is one of the symptoms of an inflammatory process. In particular, middle ear inflammation is associated with high concentrations of pro-inflammatory cytokines IL-1beta, IL-6 and TNF-alpha [4, 5], which may play an important role in the pathogenesis of OME, a disease characterised by the increased secretion of mucin [12, 13]. It has been shown that TNF-alpha induces mucus secretion in human airway epithelial cells in culture [14, 15], and that IL-6 induces mucin release in human intestinal cells in culture [16].

Previous investigations by our group have demonstrated the expression of two mucin genes, MUC5AC and MUC5B, by middle ear mucosa [7, 10], and that TNF-alpha up-regulates the secretion of MUC5AC mucin [8].

This study describes the differential influence of pro-inflammatory cytokines IL-6 and TNF-alpha on MUC5B and MUC5AC mucin secretion in HT29-MTX cells and shows the complex dynamic relationship between cytokine-induced mucus secretion.

We selected for our investigation the human mucin-secreting HT29-MTX cell line for two reasons: (1) these cells, adapted with 10^{-6} M methotrexate (MTX), form a homogeneous monolayer of goblet cells [17]. OME is characterised by the differentiation of basal cells into goblet cells [18] and by metaplastic change of the middle ear epithelium to a secretory type with proliferation of goblet cells [19]; (2) HT29-MTX cells secrete MUC5AC and MUC5B [17, 20] - two mucins that have been identified in middle ear mucosa and mucus secretions [7, 10]. For the above reasons the HT29-MTX cell line can be used to investigate the effects of cytokines on the secretion of OME-related mucins. However, because this is a colon cancer derived cell line we can not completely rule out the possibility of some metabolic differences from goblet cells in the middle ear.

The secretion of mucins in HT29-MTX cells is a growth-related phenomenon [17, 21], and allowed us to investigate the changes in cytokine-induced mucin secretion in two stages of cellular growth (pre-confluent and post-confluent).

Both pro-inflammatory cytokines, TNF-alpha and IL-6, stimulated mucin secretion in HT29-MTX cells, but in a different manner. TNF-alpha stimulated a significant increase in mucin secretion within the first hours of exposure, whereas the effect of IL-6 on mucin release was observed later and was lower in compared to TNF-alpha-induced mucin secretion, but persisted longer (Figure

3). The different responses of HT29-MTX cells to IL-6 and TNF-alpha can most likely be explained by the involvement of different signal transduction pathways for these cytokines [22, 23], the specificities in regulation of transcription of MUC5AC and MUC5B genes and posttranscriptional mechanisms, involved in the regulation of mucin synthesis and secretion. Recent studies of 5' flanking regions of MUC5AC and MUC5B genes have identified NF-kappaB binding sites within the functional promoter regions for both genes [24, 25]. NF-kappaB regulates the transcription of an exceptionally large number of genes, particularly those involved in immune and inflammatory responses [26], and TNF-alpha mediates its proinflammatory functions through the complex intracellular signalling pathway leading to activation of NF-kappaB transcription factor [27-29]. As NF-kappaB binding sites have been found within functional promoter regions of MUC5AC and MUC5B genes, and TNF-alpha stimulated MUC5AC and MUC5B mucin secretion, we can suggest that activation of the NF-kappaB transcription factor might be involved in the TNF-alpha up-regulation of MUC5AC and MUC5B gene expression.

Although the mechanism of TNF-alpha-mediated regulation of mucin secretion remains unclear, investigations in recent years have presented some details of the molecular-biological pathways of TNF-alpha-induced mucin gene expression and subsequent mucin synthesis. It has been shown [15], that TNF-alpha stimulates mucin secretion through activation of phosphatidylcholine-specific phospholipase C (PC-PLC) and subsequent release of 1,2-diacylglycerol (DAG), leading to protein-kinase C activation. DAG production by a TNF-alpha-responsive PC-PLC is pivotal to TNF-alpha-induced NF-kB activation [27]. Consequently, the NF-kappaB involvement in the TNF-alpha-induced up-regulation of mucin expression is very likely. Another study has demonstrated the participation of TNF-alpha in post-translational mechanisms of mucin synthesis. It was found that, TNF-alpha regulates MUC5AC expression by increasing the stability of the corresponding mRNA transcript [30]. The molecular mechanisms of IL-6-induced mucin secretion need further investigation.

Both cytokines (TNF-alpha and IL-6) induced high levels of MUC5AC secretion and exposure to TNF-alpha resulted in a MUC5AC hypersecretion from HT29-MTX cells (56% above the control). The levels of cytokine-induced MUC5B secretion remained low in all experiments. Our results suggest different induction of MUC5AC and MUC5B mucin genes. These observations are in

agreement with recent studies on the regulation of MUC5AC and MUC5B mucin secretion in human lung cells NCI-H292 [30, 31] and mucin gene expression in human colonic cell lines [32]. It has been shown that MUC5AC mRNA levels significantly increased after exposure to acrolein, inflammatory mediators (prostaglandin E_2 and TNF-alpha) [30], and exposure to phorbol 12-myristate 13-acetate (PMA), an activator of protein-kinase C [32], but the level of MUC5B mRNA was only slightly affected under the same conditions. All of these results suggest that the MUC5AC and MUC5B genes, although sited in the same cluster at chromosome location 11p15.5 [33], and having common sites for transcription factors within functional promoter regions [24, 25], are unlikely to be controlled in the same manner.

One interesting observation in our study was the suppressive effect of mucus on the cytokine-induced mucin secretion in HT29-MTX cells. The most likely explanation for this phenomenon is formation of visible mucus gel on the surface of the cell monolayer at the stage of confluence. Mucus gel might hinder the interactions between cytokines and their receptors on the surface of the cells and decrease the cytokine-induced mucin secretion.

In summary, we found that the up-regulating effect of pro-inflammatory cytokines IL-6 and TNF-alpha on mucin secretion in HT29-MTX cells is time-dependent, dose-restricted and depends on cell growth.

The differences in the dynamics of TNF-alpha-induced and IL-6-induced mucin secretion allow us to suggest a role for TNF-alpha and IL-6 in the pathogenesis of OME disease. Because of the high and rapid stimulation of mucin secretion, TNF-alpha could be important in the first stage of the acute inflammatory process in the middle ear. IL-6 stimulates less, but prolonged mucin secretion, and might be responsible for maintaining the inflammation. Our findings would fit with the recent studies measuring TNF-alpha and IL-6 concentrations in acute otitis media [34, 35].

CONCLUSION

Our results show that MUC5AC mucin is highly inducible but MUC5B mucin is less inducible. MUC5AC is therefore able to provide a rapid response to inflammatory mediators and would be involved in the acute inflammatory process in the middle ear. The lesser induction of MUC5B secretion may mean

that MUC5B could be responsible for the maintenance of mucin synthesis and secretion in chronic middle ear inflammation. This hypothesis is supported by the presence of MUC5B in the human middle ear with chronic otitis media [10, 36].

The investigation of cytokine-induced mucus secretion would increase our understanding of the mucosal immune response accompanying many types of acute and chronic inflammatory processes.

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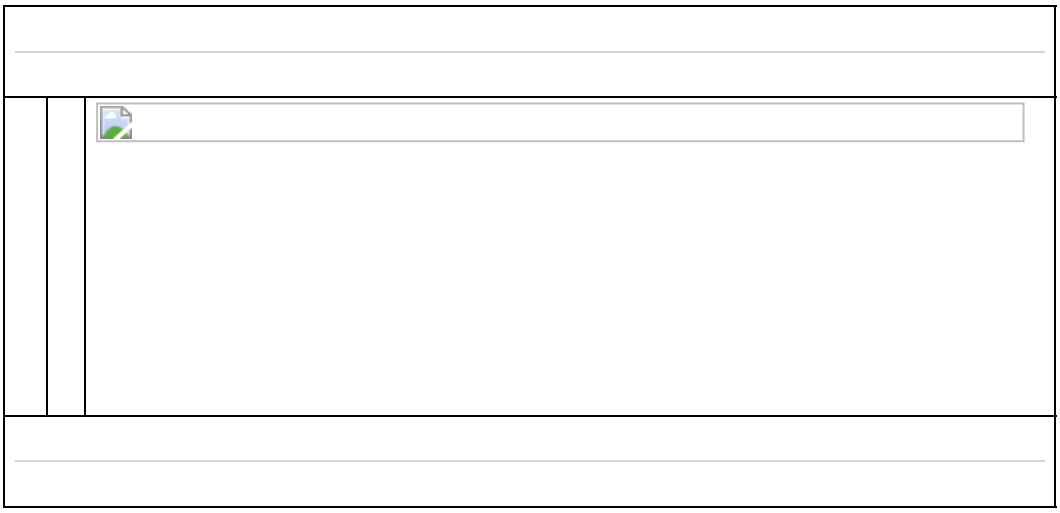
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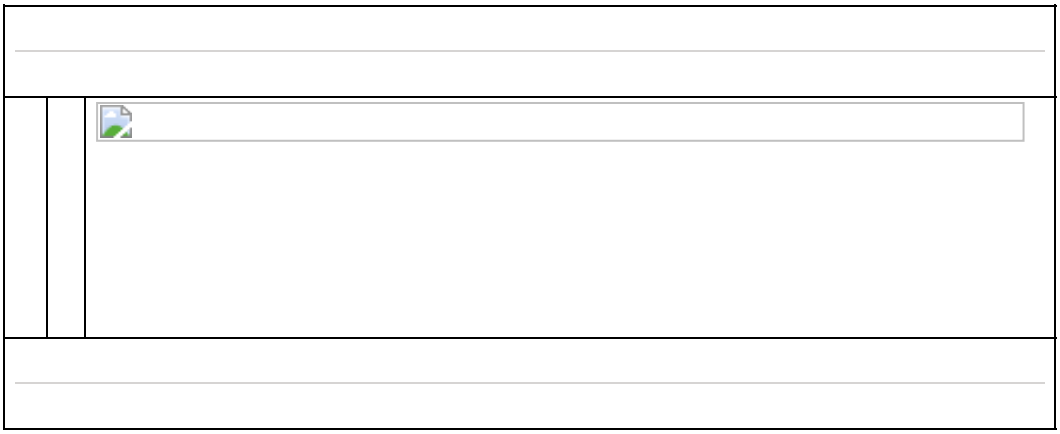
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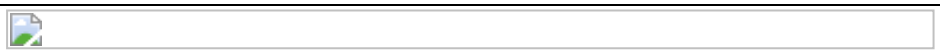




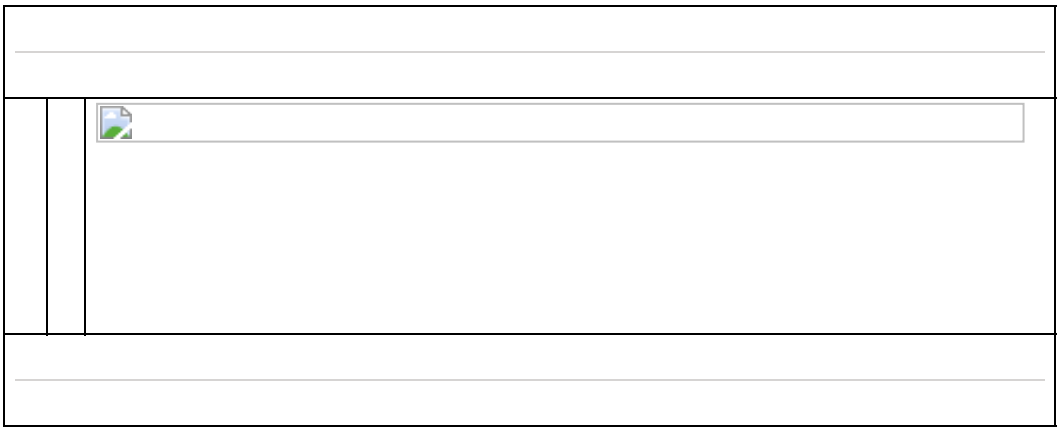












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IL-10, recombinant, rat, cytokines.

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