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Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1beta, interleukin-6 and interleukin-8 in the pathogenesis of the otitis media with effusion

Abstract

Inflammation in the middle ear mucosa, caused usually by bacterial and viral pathogens, is the primary event in the middle ear predisposing the development of otitis media with effusion (OME). Numerous inflammatory mediators have been identified in OME. However, cytokines play a central role as initiators,

mediators and regulators of middle ear inflammation and subsequent molecular-pathological processes in middle ear tissues, leading to histopathological changes in the middle ear cavity and the pathogenesis of OME. In this article, we aim to present an overview of current research developments in the pro-inflammatory cytokine involvement in the aetiology of otitis media with effusion.

Full text

INTRODUCTION

The pro-inflammatory cytokines TNF-alpha, IL-1beta, IL-6 and IL-8 are important mediators in immune regulation and are produced during immune and host defence responses.

Tumor necrosis factor (TNF-alpha) is a pleiotropic cytokine, produced primarily by macrophages in response to bacterial and viral pathogens. The pro-inflammatory functions of TNF-alpha include up-regulation of endothelial cells adhesion molecule expression, stimulation of cytokine expression, activation of neutrophils, stimulation of fibroblast proliferation, and antiviral effects against both DNA and RNA viruses [1, 2].

IL-1beta is recognized as a central mediator of inflammation, produced mainly by activated macrophages [3, 4]. IL-1beta has a wide range of target cells, including fibroblasts (causing proliferation and inducing secretion of collagenases and IL-6); endothelial cells (induces TNF-alpha release and adhesion molecule expression); osteoclasts (activates the proton pump, leading to bone resorption); mature T cells (induces proliferation of Th2 cells); mature B cells, (induces proliferation and immunoglobulin secretion); monocytes and neutrophils (induces secretion of several cytokines, including IL-8 and IL-1 itself) [5, 6].

IL-6 possesses pleiotropic activities, which play a central role in host defence. These activities include maturation of, and immunoglobulin secretion by B cells, osteoclast activation, induction of monocyte differentiation into macrophages, activation of T cells, stimulation of hepatic acute-phase protein synthesis, maturation of megakaryocytes [7, 8].

IL-8 is related to the chemokine family of cytokines. IL-8 primarily attracts and activates neutrophils and also induces the directional migration of monocytes, T lymphocytes and basophils; it releases intracellular enzymes from neutrophils and histamine from basophils; it regulates the adhesion of neutrophils and plays a key role in the accumulation of leukocytes at sites of inflammation [9, 10].

Reflecting their biological activities, TNF-alpha, IL-1beta, IL-6 and IL-8 are involved in different inflammatory reactions, accompanying the host immune response to microbial and viral infections and participate in the pathogenesis of related diseases [11-13]. Investigations over the last two decades demonstrate the involvement of TNF-alpha, IL-1beta, IL-6 and also IL-8 in the pathogenesis of otitis media with effusion (OME).

OTITIS MEDIA WITH EFFUSION - HISTOPATHOLOGICAL, CELLULAR AND MOLECULAR CHARACTERISTICS

Otitis media with effusion is the commonest cause of childhood deafness in the developed world. The disease is an inflammatory condition of the middle ear cleft and characterized in the early stage by inflammatory cell infiltration in the submucosal layer, cell proliferation in the epithelial and mucosal linings of the middle ear cavity, and accumulation of neutrophils, macrophages and lymphocytes in the middle ear fluid [14, 15]. Later accumulation of effusion in the middle ear cleft occurs as a result of mucociliary transport system dysfunction [16, 17]. In the late stage of the disease, severe and sometimes irreversible tissue pathology can develop that includes: fibrosis [18], granulation tissue, osteitis, osteoneogenesis, cholesteatoma [19], and finally histological degeneration of the tympanic membrane as a result of tissue proliferation in subepithelial and submucosal layers [20, 21], leading to hearing loss.

Otitis media (OM) can be subdivided into 4 main types: purulent (acute) (POM), serous (SOM), mucoid (MOM) and chronic (COM) [19]. These subdivisions have specific histological (Figure 1) and clinical characteristics. The symptoms of purulent otitis media include pain, fever, hearing loss and occasional perforation of the tympanic membrane.

Serous and mucoid OM are less severe, without the pain and fever of POM. However, hearing loss is present and changes in the tympanic membrane do occur. SOM and MOM frequently develop from eustachian tube dysfunction and

appear to occur in a continuum. In the early stage of otitis media, serous transudate from vessels in the subepithelial space (SES) can pass to the middle ear cleft, leads to and forms the serous otitis media [19]. There is no evidence of secretory cell proliferation in SOM [22].

Mucoid otitis media is characterized by differentiation of basal cells into goblet and ciliated cells [19], proliferation of goblet cells [22, 23], and formation of mucus and secretory gland populations [24, 25] in the middle ear mucosa. These changes lead to the increased secretory activity of the middle ear mucosa and promote the appearance of a thick mucoid effusion in the middle ear cleft. However, with time, degeneration of the new glands and a decrease in basal cells in the middle ear mucosa may occur, and the middle ear epithelium returns to normal.

Chronic otitis media can also be a painless process without fever. As in all other types of OM, hearing loss and tympanic membrane changes are the main clinical signs of COM [19]. However, at the histological level, irreversible tissue pathology is observed in COM (Figure 1). COM frequently is associated with the presence of increased numbers of mast cells in the middle ear cleft tissues [26-28]. An overlap of the histopathological findings between the different types of otitis media is characteristic (Figure 1). In addition to the main types of otitis media, there are also intermediate types such as mucopurulent and mucoserous.

Mucins are important glycoproteins in the mucociliary transport system of the middle ear and are the main component of middle ear effusions, responsible for the viscous properties of effusions [31, 32]. Middle ear mucins are able to bind to proteins in the outer membrane of bacteria [33], and play an essential role in evacuating pathogens from the middle ear. However, under disease conditions, alterations that occur in the middle ear and eustachian tube mucin metabolism [34], in the structure of mucin glycoproteins [35, 36], and in glycoconjugate expression in cilia and goblet cells [37], can contribute to dysfunction of the normal mucociliary transport system and promote the formation of effusion in the middle ear cleft.

Three mucins, namely membrane-bound MUC4 [34] and secreted MUC5AC [36] and MUC5B [34, 36, 38, 39] are found in the middle ear mucosa and mucus secretions from patients with chronic and mucoid otitis media. MUC5B is

considered to be a major mucin in the middle ear mucosa and effusions. Expression of the MUC5B mucin gene in pseudostratified middle ear mucosal epithelia correlates with the expression of inflammatory molecules ICAM-1 (intercellular adhesion molecule-1) and RANTES (regulated upon activation, normal T cell expressed and secreted) [40], and with infiltration of inflammatory cells in the submucosa of the middle ear cleft [39]. These correlations suggest that inflammation may initiate and maintain the hypersecretory state of the middle ear mucosa leading to the chronicity of OME.

It is important to note that acute inflammatory changes are usually seeing in purulent and serous OM, and chronic inflammatory changes are more severe in mucoid and chronic OM [29]. However, the overlaps in histopathological findings between different types of otitis media [29, 30] suggest that all known categories of otitis media (purulent, serous, mucopurulent, mucoserous, mucoid and chronic) can represent different stages in a continuum of events, accompanying one disease - the otitis media with effusion.

INFLAMMATORY MEDIATORS IN OTITIS MEDIA WITH EFFUSION

The molecular background explaining the pathology of OME is still unclear. However, the crucial process in the middle ear leading to OME is local inflammation. Bacteria [41-43], viruses [44-46] and allergic reactions [47, 48] are all implicated as initial stimuli of middle ear inflammation.

Different groups of inflammatory mediators, regulating different stages of the inflammatory response, are identified in the human middle ear mucosa, fluids (MEF) and effusions (MEE).

The early mediators of inflammation, initiating acute inflammatory reactions, are detected in OME and include arachidonic acid metabolites (prostaglandin PGE_2 and leukotrienes $LT\text{-}B_4$, $LT\text{-}C_4$) [49, 50], histamine [51, 52], platelet-activating factor (PAF) [53], surface cell adhesion molecules (intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1), platelet endothelial cell adhesion molecule (PECAM)) [54, 55] and the pro-inflammatory cytokines TNF-alpha [56], IL-1beta [57], IL-8 [58].

The mediators of the ongoing inflammatory process are also identified in OME and include soluble cell adhesion molecules (sICAM-1 and sVCAM-1) [59, 60], chemokine RANTES [61, 62], complement C3a anaphylatoxin [63], interferon-gamma [64, 65] and the pro-inflammatory cytokine IL-6 [66].

The immunoregulatory cytokines IL-2 [64, 67] and IL-5 [68] and cytokines that downregulate the inflammatory response, such as transforming growth factor-beta (TGF-beta) [69] and IL-10 [70], are also present in OME.

All the aforementioned inflammatory mediators are involved in the pathogenesis of OME. However, the pro-inflammatory cytokines TNF-alpha, IL-1beta, IL-6 and IL-8 are very likely to play a central role in middle ear inflammation and in the stimulation of the molecular-pathological background of OME. These cytokines are detected at high rates in middle ear effusions and are present in effusions at high concentrations. Rates and the highest mean concentration values are as follows: (63%-91%) and 234.2 ± 109.1 pg/mg of total protein (TP) for TNF-alpha [64, 71-73]; (51%-97%) and 4076 ± 1510 pg/mg of TP for IL-1beta [64, 71-73]; (36%-83%) and 173.9 ± 74.7 pg/mg of TP for IL-6 [66, 72]; (92%-100%) and 4805 ± 913 pg/mg of TP for IL-8 [71, 73].

TNF-alpha **IN OTITIS MEDIA WITH EFFUSION**

TNF-alpha was one of the first pro-inflammatory cytokines identified in middle ear effusions from children with chronic otitis media with effusion (COME) [56, 64]. Concentrations of TNF-alpha in effusions correlate with age such that the older the child, the higher the level of TNF-alpha, and the highest concentrations of TNF-alpha (up to 234 pg/mg of TP) are associated with a history of multiple placements of tympanostomy tubes [64, 72]. Children undergoing tympanostomy on multiple occasions have average levels of TNF-alpha, nearly 14 times higher than those in children undergoing their first tympanostomy [64].

The presence of TNF-alpha in otitis media correlates with the presence of bacteria [72, 74], viruses and cell adhesion molecules [54] in effusions. TNF-alpha is detected in all types of otitis media: purulent [72, 74, 75], mucopurulent [76], serous [77], mucoid [56, 73, 78] and chronic [64, 71, 72]. High concentrations of TNF-alpha (up to 67 pg/mg of TP) in mucoid effusions [73], suggest the involvement of TNF-alpha in middle ear mucin hypersecretion, one

of the important molecular-pathological processes occurring in OME. This suggestion is supported by investigations of the effects of TNF-alpha on mucin gene expression and mucus secretion in the middle ear with *in vitro* and *in vivo* models [79, 80]. The results of these investigations are discussed below.

It is important to note that TNF-alpha soluble receptor (TNF-alphasolR) has been identified together with TNF-alpha in COME [81]. The correlation between TNF-alpha and TNF-alphasolR concentrations in effusions suggests that the chronic inflammation seen in COME may be the result of an imbalance in the ratio between TNF-alpha and its natural inhibitor - TNF-alpha soluble receptor.

Although TNF-alpha is detected in different types of otitis media and effusions, the level of TNF-alpha is always higher in the chronic stage of the disease and therefore, TNF-alpha is considered to be a marker for OME chronicity [72] and related to the persistence of OME [78].

The role of TNF-alpha in the pathogenesis of OME has been studied with *in vitro* and *in vivo* models.

In vitro studies show that TNF-alpha up-regulates the expression of RANTES in cultured rabbit middle ear epithelium [62], and stimulates the secretion of mucous glycoprotein (MGP) in cultured chinchilla middle ear epithelial cells [79].

For *in vivo* studies, investigators have used two types of animal models. In the first model, recombinant TNF-alpha was directly injected into middle ears of experimental animals and subsequent immuno-histopathological changes were analyzed [80, 82, 83]. In the second model, the TNF-alpha-related processes in otitis media were analyzed after injection of bacteria or bacterial products (endotoxin) into middle ears of experimental animals [84-89].

Numerous *in vivo* studies have presented the following observations. TNF-alpha inoculated into the middle ear cavity of specific pathogen-free rats markedly increases Muc2 mucin mRNA expression in the middle ear epithelium and MGP secretion in the middle ear fluid [80].

Transtympanical injection of TNF-alpha induces acute experimental OME in the guinea pig [82] and in the rat models [83]. The inflammatory cellular effusion, containing up to 67% lymphocytes, develops within 24 hours after injection of

TNF-alpha [82]. The TNF-alpha-induced middle ear effusion is accompanied by histopathological changes such as subepithelial edema, marked infiltration of neutrophils and increased microvascular permeability in the middle ear cleft. These immuno-histopathological changes are significantly reduced by the TNF-alpha soluble receptor type I [83].

Hypersecretion of TNF-alpha (up to 200 ng/ml) is detected in experimental endotoxin-induced OME [84], and interestingly, the histhopathological changes in the middle ear caused by endotoxin are reduced significantly by the injection of TNF-alpha-binding protein [85].

TNF-alpha is present in the middle ear mucosa, fluids and effusions in experimental otitis media induced by the heat-inactivated [86] or viable, Gram-positive bacterium *Streptococcus pneumoniae* [87,88] and Gram-negative bacterium *Haemophilus influenzae* [89]. Up-regulation of TNF-alpha mRNA expression in the middle ear mucosa occurs at 6 hours after bacterial inoculation [87]. However, secretion of TNF-alpha is detected in MEF earlier, within the first 2 hours after inoculation of the pathogens and afterwards shows peaks at 6 hours, 48 hours, 72 hours and 96 hours [88, 89]. Concentrations of TNF-alpha in effusions correlate significantly with bacterial product [86], and in MEF, with the number of lymphocytes, macrophages and neutrophils [88], suggesting that TNF-alpha activates inflammatory cells in middle ear inflammation.

Investigations of TNF-alpha in human and experimental OME lead to the following conclusions:

1) TNF-alpha is produced in the early stage of inflammation by the middle ear mucosa and in the late stage by accumulating inflammatory cells, and can be considered as the primary cytokine in OME.

2) TNF-alpha correlates with the number of inflammatory cells and the presence of cell adhesion molecules in otitis media. In the middle ear mucosa TNF-alpha stimulates the expression of RANTES - one of the markers of ongoing inflammation. Therefore, TNF-alpha is a mediator of ongoing inflammation in the middle ear and is involved in the pathogenesis of purulent (acute) otitis media.

3) TNF-alpha up-regulates mucin gene expression and mucus secretion in the middle ear and probably plays an essential role in the pathogenesis of mucoid otitis media.

4) TNF-alpha induces severe histopathological changes in the middle ear tissues and can contribute to chronic OME.

5) TNF-alpha is one of the primary cytokines in otitis media induced by bacterial pathogens, both Gram-positive and Gram-negative, and participates in viral OME.

6) TNF-alpha soluble receptor may be a natural inhibitor of OME.

Thus the pro-inflammatory cytokine TNF-alpha is one of the primary mediators in middle ear inflammation (Figure 2), it regulates many molecular-pathological processes in the middle ear, and can be considered as the key cytokine involved in the aetiology of OME.

IL-1beta IN OTITIS MEDIA WITH EFFUSION

IL-1beta, as well as TNF-alpha, is present in all main types of otitis media: purulent (acute) [57, 75, 78, 90], serous [57, 77], mucoid [73, 78] and chronic [64, 72]. Purulent effusions (POM) contain the highest levels of IL-1beta, while serous (SOM) and mucoid (MOM) have the lowest [57].

In acute otitis media, the concentrations of IL-1beta in MEF with viable bacterial pathogens (culture-positive) are 3 times higher, than in culture-negative ones, and usually, the concentration of IL-1beta decreases with the eradication of bacterial pathogens following antibiotic therapy [90]. IL-1beta is also present in middle ear effusions infected with viruses [54]. These observations show that IL-1beta is a mediator of both bacterial and viral otitis media.

Investigations of IL-1beta in MEE have found the following correlations: (1) IL-1beta correlates inversely with the age of children, such that younger groups have higher levels of IL-1beta than older groups [57, 64, 75]. (2) High level of IL-1beta in the purulent effusions correlates with high level of total collagenase [75]. (3) The immuno-cytological analysis of effusions shows the highest level of IL-1beta in the neutrophil-rich effusions [78]. (4) Effusions that contain IL-

1beta also contain TNF-alpha, IL-6 and IL-8 [64,70-73]. (5) Statistically significant correlations are found between the concentrations of IL-1beta and TNF-alpha [63, 70, 72], IL-1beta and IL-6 [72], IL-1beta and IL-8 [70, 71] in effusions.

These findings present indirect evidence for a wide range of molecular processes, which IL-1beta can stimulate during inflammation in the middle ear (Figure 2), namely, up-regulation of primary (TNF-alpha and IL-1beta itself) and secondary (IL-6 and IL-8) pro-inflammatory cytokine secretion; activation of neutrophils in the zone of inflammation; fibroblast activation with subsequent up-regulation of collagenase secretion.

The role of IL-1beta in OME has been studied *in vivo*, applying two approaches.

In the first approach, recombinant IL-1beta (rIL-1beta) was transtympanically injected into experimental animals. Analysis of the pathological changes in the middle ear showed the following results. rIL-1beta (100 U) does not produce significant effusions in normal guinea pigs [82]. However, in the murine model (specific pathogen-free male BALB/c mice) of OME, rIL-1beta (100 ng) induces a middle ear effusion within three days of injection [91].

In the second approach, IL-1beta -related processes were analyzed in otitis media provoked by injection of bacterial pathogens into the middle ear cleft of experimental animals, with the following results: (1) IL-1beta is the earliest pro-inflammatory cytokine detected in MEF, less than 1 hour, after inoculation of viable pneumococci, in the chinchilla model of OME [88]. The level of IL-1beta peaks twice, at 6 hours after inoculation and before appreciable inflammatory cell accumulation, and at 24 hours, when the level of inflammatory cells is high. (2) The concentration of IL-1beta in MEF correlates significantly with the number of neutrophils, suggesting that IL-1beta activates neutrophils and induces up-regulation of its own secretion by activated neutrophils in middle ear inflammation. Correlations between the concentration of IL-1beta and the number of macrophages and lymphocytes are less significant as compared with neutrophils [88]. (3) The significant correlations between the level of IL-1beta and levels of TNF-alpha, IL-6, IL-8 and the dynamics of the secretion of these cytokines in experimental otitis media [88], suggest that IL-1beta can up-regulate TNF-alpha, IL-6 and IL-8 secretion in middle ear inflammation. (4) High levels of IL-1beta are detected in middle ear effusions in experimental otitis

media induced by both Gram-positive (heat-killed *Streptococcus pneumoniae*) [86] and Gram-negative (viable *Haemophilus influenzae*) bacteria [89]. (5) Up-regulation of IL-1beta mRNA expression occurs in the middle ear mucosa in experimental OME induced by the endotoxin [91]. Pathological changes in the middle ear stimulated by endotoxin [91] are inhibited by the anti- IL-1 receptor antibody, suggesting an important role for IL-1beta in endotoxin-induced OME.

Study of IL-1beta in human and experimental OME have produced the following conclusions:

1) IL-1beta is the earliest cytokine in middle ear inflammation, produced in the early stages of inflammation by cells in middle ear mucosa, and later by accumulated inflammatory cells, and can be recognized, together with TNF-alpha, as the primary cytokine in OME.

2) IL-1beta induces secretion of pro-inflammatory cytokines (IL-1beta itself, TNF-alpha, IL-6, IL-8) in middle ear tissues and thus stimulates the pro-inflammatory cytokine network in middle ear inflammation (Figure 2).

3) IL-1beta activates neutrophils and lymphocytes in middle ear inflammation and promotes inflammatory cells infiltration into middle ear.

4) IL-1beta is involved in otitis media induced by viral and both Gram-positive and Gram-negative bacterial pathogens.

In general, IL-1beta can be considered to be a mediator of acute and ongoing inflammation in the middle ear, associated with the early stages of the disease and the purulent (acute) type of otitis media. However, IL-1beta can induce fibroblast activation and proliferation, growth of osteoclasts in the middle ear tissues, with subsequent pathological changes such as fibrosis and bone erosion and therefore can contribute to the pathogenesis of the chronic type of OME.

IL-6 IN OTITIS MEDIA WITH EFFUSION

IL-6 has been identified in chronic OME, together with the primary pro-inflammatory cytokines IL-1beta and TNF-alpha [66, 72], and in purulent (acute) OM [92, 93]. IL-6 is detected in both bacterial and non-bacterial, acute otitis

media [92], and mRNA for IL-6 is also present in middle ear effusions and mucosal tissues, infected with respiratory syncytial virus [54].

Studies of IL-6 in middle ear effusions revealed the following. (1) Like IL-1beta, levels of IL-6 are higher in young children [66]. (2) Level of IL-6 in purulent otitis media is higher as compared to chronic otitis media, and the presence of IL-6 in biopsy specimens correlates positively with the presence of macrophages and B cells [93]. (3) In bacterial otitis media, IL-6 concentrations are higher in the culture-positive samples as compared to culture negative ones, and the concentration of IL-6 in MEF decreases significantly after antibiotic therapy [92]. (4) Concentrations of IL-6 in effusions, correlate positively with concentrations of IL-1beta and TNF-alpha [72].

These observations suggest the participation of IL-6 in the regulation of acute and ongoing stages of middle ear inflammation, and involvement of IL-6 in bacterial otitis media. Investigations of IL-6 in experimental pneumococcal otitis media [87, 88, 94] confirm this suggestion.

IL-6 is detected in MEF in pneumococcal otitis media as early as 2h after bacterial middle ear challenge [88]. Then the level of IL-6 decreases and increases a second time with two peaks, one at 48 and one at 72 hours. These changes in the level of IL-6 correlate with the inflammatory cell concentration, suggesting that IL-6 is produced by the accumulated inflammatory cells [88]. Transcripts of IL-6 (mRNA) have been observed in purulent effusions and in the middle ear mucosa only 24 hours after inoculation of pathogen, however, the IL-6 cytokine (protein) persisted in middle ear tissues for at least 5 days following bacterial injection [94]. IL-6 is localized mainly in the mucosal epithelium, but also in the deeper layers of the tissues such as the bullular bone, deep in the submucosa and around blood vessels [94].

The concentration of IL-6 in MEF correlates significantly with the number of inflammatory cells (especially macrophages and lymphocytes), and primary cytokines (IL-1beta and especially TNF-alpha) [88]. It is important to note that up-regulation of IL-6 mRNA (peak at 12 to 24 hours), in experimental pneumococcal otitis media, occurs after up-regulation of TNF-alpha expression (within 6 hours after inoculation of pathogens) [87].

Investigations of IL-6 in human and experimental OME led to the following conclusions:

1) IL-6 is a mediator of middle ear inflammation, and is produced by cells in the middle ear mucosa and by the accumulated inflammatory cells (mainly macrophages and possibly T cells).

2) IL-6 activates B cells in middle ear inflammation.

3) The secretion of IL-6 during middle ear inflammation is under control of the primary pro-inflammatory cytokines IL-1beta and TNF-alpha, and IL-6 can be considered as the secondary cytokine in middle ear inflammation (Figure 2).

4) IL-6 is involved in otitis media stimulated by both bacterial and viral pathogens.

In general, IL-6 is the regulator of the ongoing inflammatory processes in the middle ear associated with the early stage of OME. However, IL-6 can initiate a differentiation of macrophages to osteoclasts and thereby participate in a bone remodeling process leading to development of tympanosclerosis and switching the disease to the chronic stage.

IL-8 IN OTITIS MEDIA WITH EFFUSION

IL-8, as well as TNF-alpha, has been identified in most types of otitis media: purulent (acute) [95, 97, 98, 99], serous [95-97], mucoserous [96], mucoid [73, 95, 97], and chronic [97]. Concentrations of IL-8 in acute otitis are significantly higher than in chronic OME [98] and mucoid OM shows significantly higher mean concentrations of IL-8 in comparison with serous OM [96, 97].

IL-8 is detected in a high proportion of effusions analyzed (up to 100%) [73], and usually shows the highest mean concentration value of an the other pro-inflammatory cytokines measured; typically an IL-8 concentration of 4,805 pg/mg is present, whereas concentrations of IL-1beta and TNF-alpha in the same effusions are 4,075 pg/mg and 163 pg/mg, respectively [71]. However, there are significant and positive correlations between the concentration of IL-8 and concentrations of IL-1beta and TNF-alpha in effusions [70, 71], suggesting that IL-1beta and TNF-alpha can induce the IL-8 hypersecretion seen in OME.

The presence of IL-8 in effusions correlates with the total number of neutrophils, and purulent effusions contain more neutrophils than mucoid and serous effusions [96]. Although IL-8 is an important chemotactic factor for leukocytes, the analysis of chemotactic activity in effusions shows that IL-8 cannot be considered as the main chemotactic component in MEE [98].

Investigations of IL-8 in MEE show that IL-8 is involved in the pathogenesis of otitis media induced by bacterial and viral pathogens. The IL-8 concentration in effusions with bacteria is higher than in effusions without bacteria [98, 99]. The decrease of the IL-8 concentration in middle ear fluid correlates with bacterial eradication from the middle ear following antibiotic treatment [99]. IL-8 mRNA is identified in effusions, together with mRNA for respiratory syncytial virus and parainfluenza virus type 3 [61].

The significance of IL-8 in the aetiology of OME has been shown with *in vivo* and *in vitro* models. Transtympanical injection of human IL-8 (25 mug/ml) induces middle ear inflammation in experimental OME [100]. The inflammation develops within the first 8 hours after IL-8 injection, and is accompanied by thickening of the epithelial layer and infiltration of the inflammatory cells into the subepithelial space.

Gram-positive and Gram-negative, middle ear bacterial pathogens, induce the IL-8 network within different types of experimental otitis media [88, 89, 101].

The secretion of IL-8 in MEF is detected in otitis media provoked by viable *Streptococcus pneumoniae* [88] and non-viable *Haemophilus influenzae* [89]. It is important to note that IL-8 is not detected in MEF before 12 hours, but is significantly increased at 48 hours, 72 hours and 96 hours after inoculation of pathogens [88, 89], suggesting secretion of IL-8 by the increased population of inflammatory cells during the later stages of middle ear inflammation. Positive and significant correlations exist between the concentration of IL-8 and number of neutrophils, but not with lymphocytes, in MEF [88], supporting the theory that IL-8 is primarily a neutrophil chemoattractant. The IL-8-related accumulation of neutrophils in the middle ear is also detected in otitis media stimulated by endotoxin [101].

The kinetics of IL-8, IL-1beta and TNF-alpha secretion during experimental middle ear inflammation caused by bacterial pathogens, and correlations

between the concentrations of these cytokines in the middle ear fluid [88, 89] provide indirect evidence that IL-1beta and TNF-alpha control IL-8 expression in otitis media.

A new gram-positive and OME-related bacterium *Alloiococcus otitidis*, and soluble extracts from the inactivated *A. otitidis*, show the ability to stimulate IL-8 secretion in cultured monocytes and epithelial cell lines [102]. Although monocytes produce significantly higher levels of IL-8 than the epithelial cells, these results demonstrate that both monocytes and epithelial cells may be the source of IL-8 in middle ear inflammation, and contribute to the IL-8 hypersecretion seen in OME.

The results of investigations of IL-8 in OME within clinical materials (middle ear effusions, fluids and mucosal biopsy samples), and *in vivo* and *in vitro* models allow us the following conclusions:

1) IL-8 is a mediator of middle ear inflammation, produced later than other pro-inflammatory cytokines (IL-1beta, IL-6 and TNF-alpha) by the accumulated inflammatory cells and possibly by cells in the middle ear mucosa.

2) IL-8 is the main chemotactic factor for neutrophils in middle ear inflammation and is responsible for the accumulation of neutrophils in middle ear effusions.

3) The primary pro-inflammatory cytokines, IL-1beta and TNF-alpha, can control the IL-8 expression and secretion during middle ear inflammation, therefore IL-8 can be considered as the secondary cytokine in middle ear inflammation (Figure 2).

4) IL-8 participates in the middle ear local immune response to viral and both Gram-positive and Gram-negative, bacterial pathogens.

5) IL-8 is present in different types of otitis media and is involved in both acute and chronic inflammatory reactions, in the middle ear.

In general, IL-8 is the mediator of the ongoing inflammatory process in the middle ear and is responsible for inflammatory cell infiltration into the middle ear tissues and accumulation of inflammatory cells in middle ear effusions.

Investigations of the pro-inflammatory cytokines in human and experimental OME demonstrate that all four cytokines TNF-alpha, IL-1beta, IL-6 and IL-8 are produced in the middle ear tissues in response to bacterial and viral pathogens and participate in stimulation and regulation of the molecular processes accompanying the middle ear inflammation (Figure 2).

ENDOTOXINS, PRIMARY CYTOKINES TNF-alpha AND IL-1beta AND INFLAMMATORY REACTIONS IN THE MIDDLE EAR

All four cytokines, TNF-alpha, IL-1beta, IL-6 and IL-8, are involved in the pathogenesis of otitis media caused by both Gram-positive [88] and Gram-negative bacterial infection [89]. However, in this chapter we are focusing on endotoxin-induced otitis media, because endotoxins (lipopolysaccharides), the major components of the outer membrane of Gram-negative bacteria, play an important role in the manifestation of Gram-negative infection [103], and endotoxins are detected in middle ear effusions together with the primary cytokines TNF-alpha and IL-1beta [72, 104]. It is important to note that endotoxins can be also released in a biologically active form during death of the microorganisms resulting from host immune mechanisms or antibiotic treatment [105].

Endotoxins are identified in middle ear effusions from patients with chronic OME [106, 107], and in the persistent effusions following acute otitis media [108, 109]. Endotoxin is not easily eradicated by local host defense mechanisms and persists in the middle ear, even after effective antibiotic treatment, for up to 3 months [104]. Endotoxins are identified in effusions more frequently than the viable bacterial pathogens [110, 111], suggesting that endotoxins by themselves (in the absence of viable bacteria), can induce inflammation and pathological changes in the middle ear. This assumption is confirmed in the experimental models of OME, where endotoxins, derived from different bacterial pathogens *(Haemophilus influenzae, Klebsiella pneumoniae)*, induce similar histopathological changes in the middle ear. These changes include: mucosal and submucosal inflammatory infiltrate containing T-lymphocytes, macrophages and neutrophils [112, 113], interstitial edema, thickened epithelium with intracellular edema [114, 115], hyperplasia of goblet cells [116, 117] and dysfunction of the mucociliary transport system resulting in the accumulation of effusions in the middle ear cavity [118, 119].

Analysis of molecular mediators of endotoxin-induced OME within *in vivo* models shows the following: 1) high concentrations of TNF-alpha (up to 200 pg/ml) in the middle ear fluid [84] and expression of IL-1beta messenger RNA in the middle ear mucosa [91] are detected in experimental, endotoxin-induced OME; 2) the endotoxin-induced pathological changes in the middle ear, such as vascular extravasation and accumulation of effusion are significantly attenuated by TNF-alpha binding protein [85] and anti-IL-1 receptor antibodies [91]. These findings demonstrate that endotoxin stimulates the local production of TNF-alpha and IL-1beta in the middle ear, and suggest that TNF-alpha and IL-1beta are the mediators of endotoxin-induced middle ear inflammation.

The statistically significant correlations between concentrations of bacterial endotoxin and the primary cytokines in the effusion samples [76, 104, 120] confirm this hypothesis.

Concentrations of endotoxin and both TNF-alpha and IL-1beta correlate positively and significantly in the culture-positive (containing viable bacteria) and in culture-negative effusions [104, 120]. Concentrations of TNF-alpha and endotoxin also correlate with the type of effusion: in effusions classified as mucopurulent, both TNF-alpha and endotoxin levels are significantly higher as compared to mucoid or serous types [76].

The adhesion molecules, intercellular (ICAM-I) and vascular (VCAM-1) are also present in effusion samples with endotoxin and primary cytokines [120], but their concentrations do not directly correlate with neither endotoxin or TNF-alpha and IL-1beta. However, both cytokines TNF-alpha and IL-1beta and, to some extent, bacterial endotoxin, stimulate ICAM-I expression in the middle ear epithelium *in vitro* [121]. It is important to note that ICAM-I is a specific cell-surface molecule. ICAM-I is expressed by epithelial, endothelial and antigen-presenting cells [122], and, being adhesive for ligands on circulating leukocytes, accumulates leukocytes at sites of inflammation [123]. The membrane-bound integrin receptors LFA-1 (lymphocyte function associated molecule-1) and Mac-1 (membrane attack complex-1) are the ligands for ICAM-I [122]. These three molecules, ICAM-I, LFA-1 and Mac-1, are markers of ongoing inflammation.

Thus, a direct link exists between the external stimulus of the middle ear inflammation - bacterial endotoxins, and the internal, primary cytokines of inflammation, TNF-alpha and IL-1beta, and ICAM-1, which is expressed on the cell

surface during inflammation. This fact gives us the opportunity to show the start of the endotoxin-induced inflammatory cascade in the middle ear, mediated by the primary pro-inflammatory cytokines (Figure 3).

Bacterial endotoxin provokes the sustained production of TNF-alpha and IL-1beta by cells (in particular, macrophages), in the middle ear mucosa. TNF-alpha and IL-1beta, in turn, induce two important molecular processes: secretion of secondary cytokine IL-8 by goblet cells and endothelial cells, and up-regulation of ICAM-1 expression on the epithelium and vascular endothelium and probably on the antigen-presenting cells. Secreted IL-8 induces chemotaxis of polymorphonuclear neutrophils (PMN), already activated by the earliest mediators of inflammation (histamine, platelet-activating factor (PAF), and leukotrienes), from the bloodstream, to the surface of endothelial cells. The interaction between ICAM-1 receptors on endothelial cells and its ligand LFA-1 on activated neutrophils binds neutrophils very firmly to the endothelial surface and facilitates the subsequent migration of neutrophils from the bloodstream into infected mucosa (Figure 3). The interaction between ICAM-I and its ligands LFA-1 and Mac-1 strengthens the binding of immunocompetent cells to antigen-presenting cells, which is a prerequisite for antigen recognition and subsequent immunostimulation in the middle ear.

CONCLUSION

Local inflammation in the middle ear mucosa is the crucial event in the development of middle ear effusion (OME). The pro-inflammatory cytokines, TNF-alpha, IL-1beta, IL-6 and IL-8, are the key mediators of middle ear inflammation. These cytokines regulate the molecular processes, which lead to the pathological changes in the middle ear in early stages of the disease such as: tissue infiltration of inflammatory cells, mucin hypersecretion, accumulation of effusion in the middle ear cleft. However, the pro-inflammatory cytokine network, probably against the background of additional predisposing factors such as eustachian tube dysfunction, obstructive adenoids, allergy and reflux, can provoke irreversible changes in the middle ear tissues and encourage chronic otitis media with effusion.

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Figures

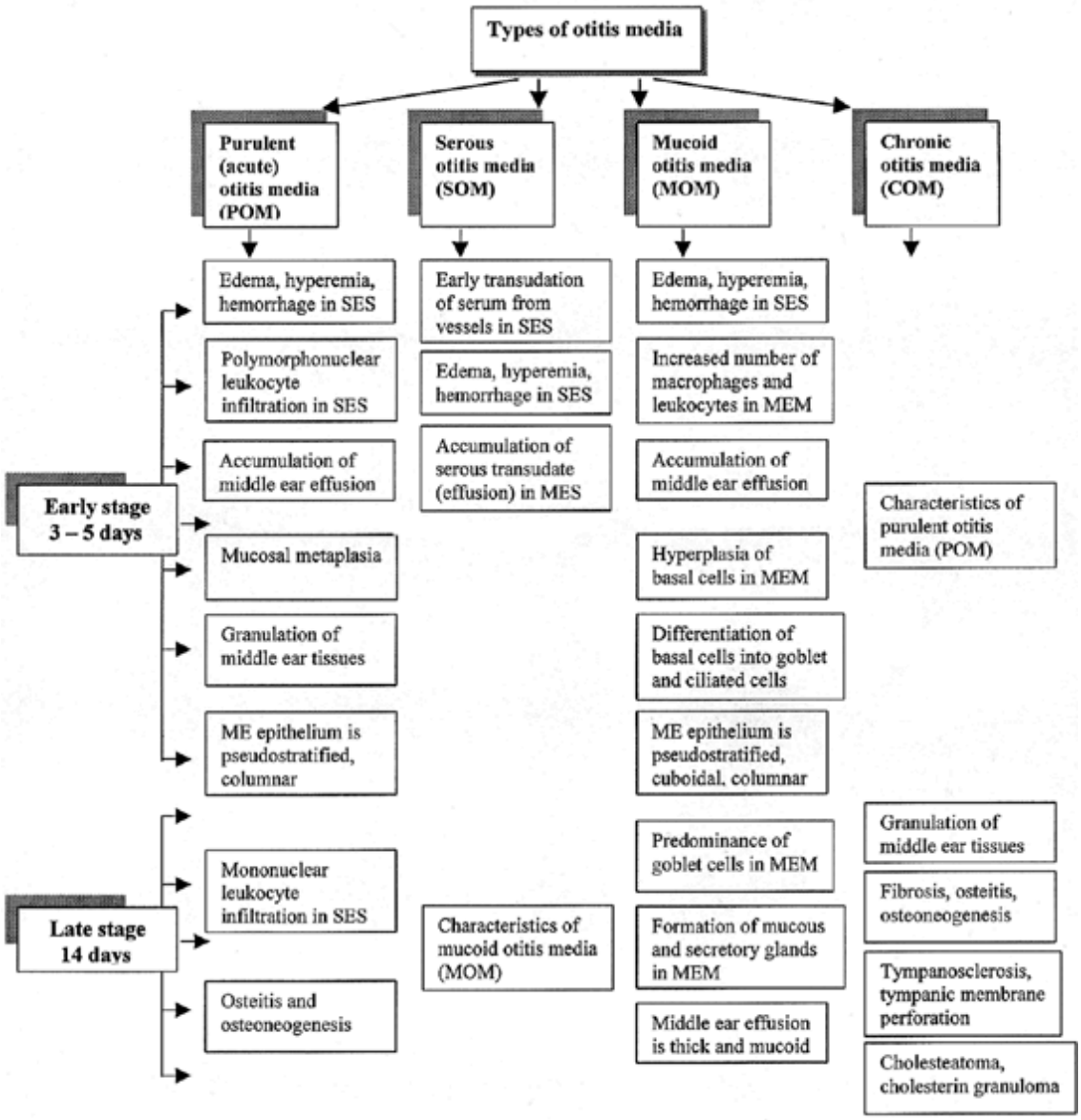


Figure 1

Histopathological characteristics of different types of otitis media.

SES: subepithelial space; ME: middle ear; MES: middle ear space; MEM: middle ear mucosa.

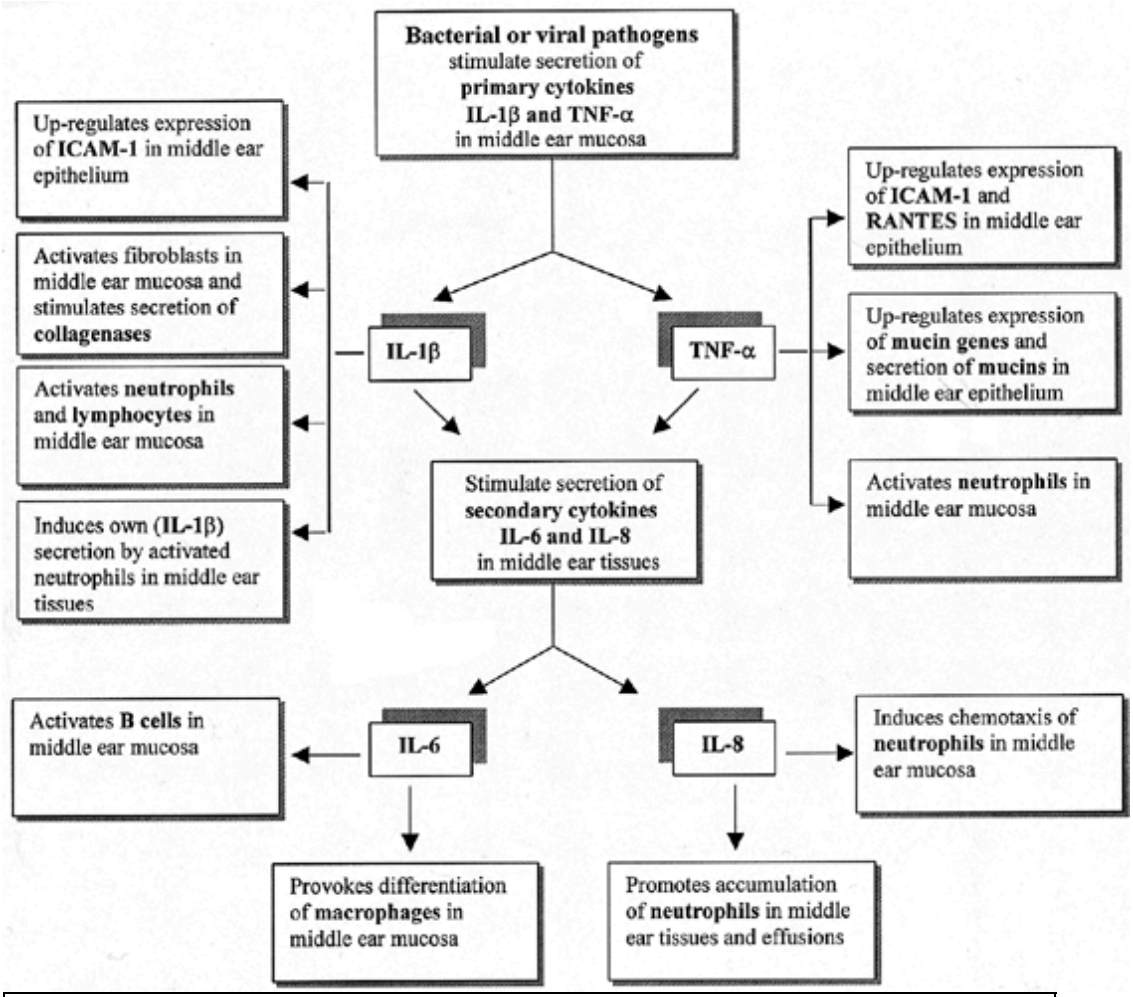


Figure 2

The pro-inflammatory cytokine network in middle ear inflammation.

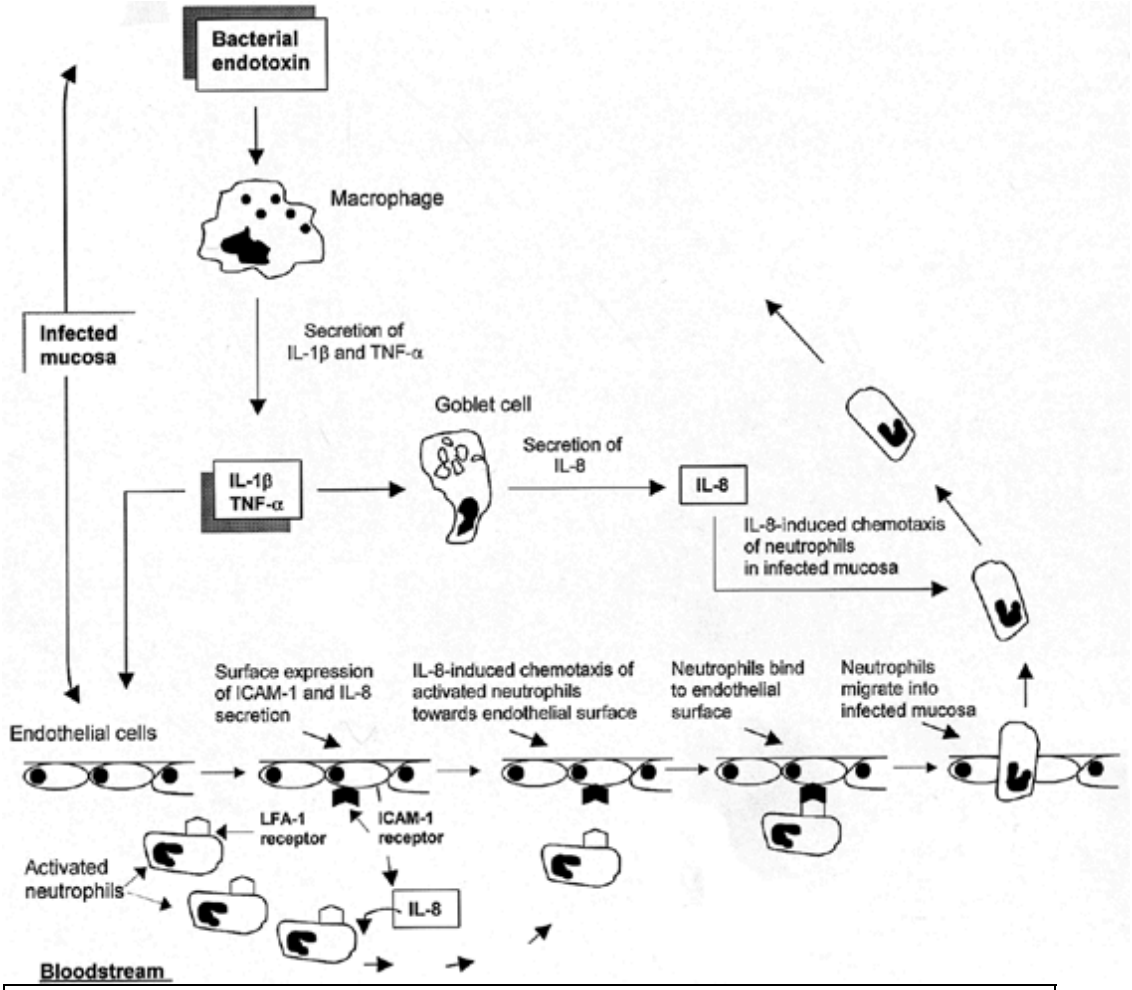


Figure 3

Endotoxin-induced inflammatory cascade in the middle ear tissues.

Authors

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