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Inhibition of Message for FcεRI Alpha Chain Blocks Mast Cell IL-4 Production Induced by Co-Culture with *Mycoplasma pneumoniae*

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Abstract

We have previously described the activation of RBL-2H3 mast cells for IL-4 production by *Mycoplasma pneumoniae* but the mechanism remains unclear. *M. pneumoniae* binds eukaryotic cells primarily through sialoglycoproteins on the target cell surface. This study was undertaken to determine whether the sialated FcεRIα chain on RBL cells is important for *M. pneumoniae*-induced IL-4 production. We found that IgE-mediated IL-4 release by a series of RBL sublines correlated with the release induced by *M. pneumoniae*. Further, aggregation of FcγRII (CD32) in RBL cells using a monoclonal antibody inhibited both IgE-mediated and mycoplasma-induced IL-4 production, providing further evidence for an Fc receptor-mediated mechanism of activation. To examine the role of FcεRI in mycoplasma-induced IL-4 release, we created stably transfected RBL sublines using a vector expressing a short hairpin sequence designed to inhibit message for the FcεRI α chain. IgE-induced IL-4 production by the transfected sublines was reduced in similar proportion to the degree of message suppression. *M. pneumoniae*-induced IL-4 production in the four transfected sublines was completely blocked in contrast to results with the controls or parent RBL cells. We conclude that the heavily glycosylated FcεRI α chain is required for activation of mast cells for IL-4 production by *M. pneumoniae*.

Keywords

Mast Cells; *Mycoplasma pneumoniae*; cytokines; Fc receptors; asthma

1. Introduction

Mycoplasma pneumoniae is a minute bacterium that lacks a cell wall and possesses a near minimal genome containing only 816,394 nucleotide pairs and 788 open reading frames [1–2]. Despite the apparent limitations suggested by its small genome, *M. pneumoniae* is a highly specialized pathogen that frequently causes upper and lower respiratory tract infections in humans, often resulting in atypical pneumonia [3]. Persistent infection is well documented in humans and animal models, and newer data confirm its ability to exist as a facultative

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intracellular pathogen [4–6], an observation which fits well with its small genome, the result of reductive genetic evolution and the signature of an intracellular organism [5].

Increasing evidence suggests that *M. pneumoniae* may also play a role in asthma [7]. Some studies have detected the presence of *M. pneumoniae* at significantly higher rates in asthmatic patients than in nonasthmatic control patients [8–12]. Treatment of asthmatics with macrolides frequently has a beneficial effect on airway reactivity, and, in the study by Kraft *et al.*, the beneficial effect was only evident in the subset of asthmatics with evidence of *M. pneumoniae* infection [13–15]. It remains controversial, assuming these associations can be demonstrated in large well-controlled studies, whether the benefit may be due in some patients to treatment of an underlying infection or some other type of anti-inflammatory effect. However, this observation suggests that, as is the case with *Helicobacter pylori*, a sizable percentage of the population may be chronically infected with *M. pneumoniae*, and in those individuals with a genetic predisposition towards allergy and airway hyperreactivity, asthma may result..

It has long been known that *M. pneumoniae* attaches to host cells through a specialized adherence organelle. One member of a complex of proteins concentrated there, the 170 kDa P1 adhesin, plays a major role in the attachment of *M. pneumoniae* to host cells [16]. Reduced P1 expression at the adhesive tip in mutant bacterial strains or blockade of P1 using monoclonal antibodies results in reduced adherence [17–19]. Sialic acid residues decorating eukaryotic cell surface glycoproteins comprise a major target for adhesion by the bacterium, accounting for its ability to adhere to a wide variety of mammalian cells and even those of birds [20–21].

Following the observation that *M. pneumoniae* induces IL-4 production in the rat mast cell line RBL-2H3, we used this cell line as a model system to investigate the molecular mechanism of cellular activation. Previous studies in our laboratory showed that the production of mRNA for IL-4, IL6, and TNF- α in RBL 2H3 cells is up-regulated after two hours co-culture with live (but not heat-killed) *M. pneumoniae*, and that IL-4 protein secretion into co-culture supernatants peaks at 4–6 hours [22]. Efficient mast cell activation requires an adhesive phenotype of *M. pneumoniae* as well as cell-cell contact. Only *M. pneumoniae* grown under plastic-adherent conditions, but not under non-adherent conditions, efficiently induces IL-4 production. Bacteria introduced into cell cultures in filter inserts were unable to induce activation, and centrifugation to pellet the organisms onto the monolayer of adherent mast cells significantly augmented cytokine production.

Our previous work has also demonstrated that the ability of *M. pneumoniae* to activate mast cells for cytokine production depends upon expression of the P1 adhesin by the bacterium and upon the presence of sialic acid residues on surface glycoproteins on the host cell [22–23]. *M. pneumoniae* grown under plastic-adherent conditions expresses more P1 than bacteria grown under non-adherent conditions, correlating with the enhanced ability of organisms grown under plastic-adherent conditions to induce mast cell IL-4 production. Further, treatment of *M. pneumoniae* with anti-P1 antibody prior to co-culture inhibited IL-4 production by over 85%, and a mutant P1-deficient strain of *M. pneumoniae* is completely unable to stimulate cytokine production. We also previously demonstrated that pretreatment of the mast cells with neuraminidase, which cleaves sialic acid residues from surface glycoproteins on the target cell surface, blocks activation of the cells by the organism, consistent with numerous observations related to the adherence requirements of the organism [20–21].

Previous studies in our laboratory demonstrated that stimulation of RBL cells with covalently cross-linked oligomeric IgE induces a very similar cytokine mRNA profile to that induced by co-culture with *M. pneumoniae*. The high affinity IgE receptor Fc ϵ RI is responsible for binding to IgE and, upon receptor aggregation, generation of intracellular signaling leading to granule

exocytosis and cytokine production [24–25]. FcεRI is composed of an α chain, which binds IgE with high affinity but lacks a cytoplasmic signaling motif, a regulatory β chain with four transmembrane domains, and a disulfide-linked homodimer of γ chains which is essential for efficient signaling by the receptor complex. Only the α chain includes N-linked glycosylation sites in its extracellular domain, and there are seven sites with aggregate carbohydrate composition contributing 38–42% of M_r [26–27]. We hypothesized that the FcεRI α sialoglycoprotein was among the targets of the adherence organelle of *M. pneumoniae* and the main one responsible for mycoplasma-induced cytokine production in this cell type. The experiments detailed in this report support this model.

2. Results

2.1. IgE-mediated mast cell IL-4 production correlates with that induced by *M. pneumoniae*

If *M. pneumoniae* activates RBL mast cells by binding FcεRIα, then the response to mycoplasma stimulation in RBL sublines with different IgE responses should vary in a similar manner. To compare the mast cell IL-4 response to IgE to that after co-culture with *M. pneumoniae*, a series of RBL-2H3 sublines were created by limiting dilution cloning. The responses of these sublines to a low concentration of oligomeric IgE (0.5 μg/ml) and *M. pneumoniae* were assessed. There was a significant correlation between the amount of IL-4 produced by 11 different RBL sublines in response to IgE and *M. pneumoniae* stimulation (Fig. 1). This result provides further support for the hypothesis that *M. pneumoniae* induces mast cell IL-4 production through the activation of FcεRI since it implies that the same signaling pathway is being utilized.

2.2. FcγRII ligation inhibits both IgE and *M. pneumoniae*-induced mast cell IL-4 production

In order to further evaluate involvement of Fc receptors in mast cell activation by *M. pneumoniae*, RBL cells were pretreated with/without purified mouse anti-rat CD32 Mab for one hour at concentrations up to 0.5 μg/mL, then stimulated with IgE, *M. pneumoniae*, or PMA/ionomycin. After four hours incubation, supernatants were checked for IL-4 production. The results demonstrate that FcγRII ligation inhibits IgE or *M. pneumoniae*-induced IL-4 production by RBL cells in a dose-dependent manner but not that produced by stimulation with PMA/ionomycin (Fig. 2).

2.3. RNAi-mediated blockade of FcεRIα chain mRNA synthesis

To investigate the role FcεRI plays in *M. pneumoniae*-induced mast cell cytokine production, we used RNAi technology to block mRNA expression of the FcεRIα chain in the RBL cell line. A stable sham-transfected subline and four sublines expressing the active construct, T153-2, T153-3, T153-8, and T153-15, as well as the parental RBL cell line, were tested by real-time PCR for evidence of FcεRIα message suppression. Real time PCR analysis was performed on cDNA created from lysates of cells cultured for 48 hrs at an initial density of 0.5×10^6 cells/well in 24 well culture plates. The FcεRIα mRNA expression levels from the sham-transfected line and untreated RBL cells were similar while those from the lines containing the active construct, T153-2, T153-3, T153-8, and T153-15 cells were all reduced (Fig. 3). The degree of mRNA inhibition correlated with the amount of green fluorescence seen in the four active transfectant sublines (data not shown). Finally, the transfected sublines were tested for their IL-4 response to FcεRI aggregation using oligomeric IgE, and the responses correlated with the measured mRNA levels for FcεRI α (Fig. 4).

2.4. FcεRIα chain suppression ablates the mast cell IL-4 response to *M. pneumoniae*

To assess whether the active siRNA construct had any effect on the RBL IL-4 response to *M. pneumoniae* stimulation, experiments were performed using the sham-transfected subline, and

the T153-2, T153-3, T153-8, and T153-15 sublines transfected with the active construct. The results demonstrate that while the sham cell line produced IL-4 in quantities similar to that produced by the parent cell line, all four cell lines containing the active construct made essentially no IL-4 response to *M. pneumoniae* infection irrespective of the degree of FcεRI α chain suppression (Fig. 5). Similar tests using three other sham-transfected control cell sublines yielded similar results (data not shown). In a further set of experiments, four stably transfected RBL sublines expressing siRNA targeting rat Toll-like receptor-2 (TLR2) did not show any difference in their response to *M. pneumoniae* from the sham transfected control cells or the parental RBL cells (data not shown). Together these data indicate that FcεRIα chains are indispensable for mediating the stimulatory signal from *M. pneumoniae* infection to induce cytokine production by RBL cells.

3. Discussion and Conclusions

The results presented here demonstrate that the heavily glycosylated FcεRIα chain is required for *M. pneumoniae*-induced mast cell IL-4 production. Although formal demonstration for direct interaction between the α chain and the organism is lacking, the known adherence properties of *M. pneumoniae*, the heavily glycosylated structure of the extracellular domain of FcεRIα, and the functional data obtained in this experimental system make it highly probable that such an interaction comprises the molecular mechanism for activation of mast cells by the bacterium. By extension, binding of immunoreceptors and other signaling molecules on the cell surface of other cell types may produce diverse activating responses, perhaps accounting for the bizarre phenomena long associated with *M. pneumoniae* infection.

The experiments performed using anti-CD32 Mab clearly support a primary role for Fcγ receptor-mediated activation as the main mechanism behind mast cell activation. FcγRIIB (CD32B) is an inhibitory Fc receptor that specifically inhibits immunoreceptor-mediated cellular activation through the action of the inositol phosphatase SHIP1 which is recruited to the Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in its cytoplasmic tail following ITIM phosphorylation by aggregation with activating immunoreceptors [28]. FcγRIIB co-aggregation with other Fcγ receptors, including FcεRI, inhibits signaling by those receptors by downregulating the signals transmitted through phosphorylation and hydrolysis of inositol phospholipids [29–31]. However, the mechanism by which inhibition in the current report occurs is unclear and apparently is previously unreported. It has been shown that IgE also binds to FcγRII and FcγRIII [33]. Thus, it is likely that the covalently coupled oligomeric IgE also incorporates CD32B into surface aggregates of FcεRI, a process which would be augmented by further aggregation by the anti-CD32 monoclonal antibody. The relatively selective inhibitory effect of FcγRIIB on immunoreceptor protein tyrosine kinase-mediated activation supports the hypothesis that activation of mast cells by *M. pneumoniae* proceeds through FcεRI. These data further support the hypothesis that *M. pneumoniae* activates RBL cells through Fc receptors, including FcεRI.

It is surprising that even though the mRNA for FcεRIα in the T153-2 and T153-3 sublines is only reduced about 30%, the corresponding mycoplasma-induced IL-4 production is almost completely blocked in a manner indistinguishable from the two sublines showing a greater degree of mRNA inhibition, T153-8 and T153–15. This was in contrast to the graded inhibition seen in response to IgE. This difference in mast cell activation for cytokine production between the ability of oligomeric IgE (a soluble, high affinity ligand) and *M. pneumoniae* (a particulate ligand) may be due to the differences in the efficiency of binding, specificity of binding (relatively nonspecific in *M. pneumoniae*), and relative amount of mast cell surface area over which binding can occur. We interpret the results to mean that even a 30% inhibition of FcεRI expression reduces below a critical threshold the chances that aggregation of individual FcεRI receptor complexes within a mass of other sialoglycoproteins along the adhesin-studded

mycoplasma attachment organelle will result in close enough proximity of pairs of receptors to result in efficient cellular activation (Fig. 6).

The identification of the mechanism by which *M. pneumoniae* activates mast cells also may be involved in some of its observed effects on cytokine production by other cell types. Among the mix of surface sialoglycoproteins tethered by the organism during attachment are likely to be a host of receptors, many of which can be activated by aggregation along the bacterial adherence organelle. Human airway cells co-cultured with the organism produce RANTES and TGF β 1 [32]. Peripheral blood mononuclear cells incubated with the organism released IL-2, TNF α , and IL-6 [33–34]. *M. pneumoniae* co-culture induced IL-8 and IL-1 production by human lung epithelial and monocytoid tumor lines [35–36]. The effects of *M. pneumoniae* in the activation of diverse cell types is compatible with a model in which the nonspecific aggregation of surface molecules during adherence to at least some cell types results in activation through different cell surface receptors.

Since *M. pneumoniae* is primarily a respiratory pathogen, its ability to activate mast cells, well known to be important in allergic asthma, as well as macrophages, respiratory epithelial cells, and lymphocytes for cytokine production provides a theoretical basis for it to act as an inflammatory stimulus. Mounting evidence supports a role for chronic infection by atypical pathogens such as *M. pneumoniae* in the pathogenesis of asthma [3,37–38]. As speculated by Martin [38], patients with chronic pulmonary infection by *M. pneumoniae* who develop asthma may have a subtle immunologic defect, and this is supported by the recent report that a loss of function allele for CCR5 is protective for the development of asthma in patients infected with *M. pneumoniae* [39].

Our data presented in this report demonstrate for the first time a specific mechanism by which *M. pneumoniae* induces mast cell cytokine production. We speculate that the ability of *M. pneumoniae* to activate mast cells may be generalizable to a variety of cells of the innate and adaptive immune system, acting in effect as a low affinity particulate lectin, capable of binding to a wide variety of cell surface signaling molecules. Activation of elements of the innate and adaptive immune system could thus be directly related to the notable occurrence of diverse inflammatory and autoimmune phenomena following infections [37].

4. Materials and Methods

4.1 Cells

The RBL-2H3 cell line, a rat mucosal mast cell tumor line, was a gift of Dr. Robert Hohman (NIH/NIHAIID). Cells were propagated in RPMI-1640 medium (Mediatech) with L-glutamine supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 U/mL streptomycin. For the mast cell stimulation assay, 0.5×10^6 cells/mL were plated at 1 mL/well in 24-well plates and incubated overnight to confluence.

The ATCC M129 strain of *M. pneumoniae* was grown to confluence in SP-4 medium in tissue culture-treated polystyrene flasks (Costar). The PBS-rinsed adherent cells were removed using a cell scraper, washed twice with PBS by centrifugation, then frozen in aliquots at -80°C . Colony forming units (CFU) per mL of frozen stock were calculated by culture of a freshly thawed aliquot.

4.2. RNAi

The target gene used in these experiments was *Fcer1a* (GenBank Accession **M21622**) and the vector was pcDNATM6.2-GW/EmGFP-miR, (BLOCK-IT®, Invitrogen cat # K4936-00). The two complementary single-stranded DNA oligonucleotides encoding the target *Fcer1a* siRNA sequence were designed and synthesized by the manufacturer. The DNA sequences used for

the siRNA construct were 5'-TGCTGTAAGTATTCTAATCCACGGTGGTTTTGGCCACTGACTGACCACCGTGG TAGAATACTTA (64bp); and 5'-CCTGTAAGTATTCTACCACGGTGGTCAGTCAGTGGCCAAAACCACCGTGGAT TAGAATACTTAC (64bp). The single-stranded oligonucleotides were annealed to generate a double-stranded oligo (ds oligo). The ds oligo was cloned by ligation into the linearized pcDNA™ 6.2-GW/EmGFP-miR vector, which also codes for constitutive GFP expression in the transfected cells. The ligation reaction was used to transform One Shot TOP10 chemically competent *E.coli*, and transformants were selected using 250 µg/mL Spectinomycin(Sigma). Plasmids were extracted and sequenced to verify the correct sequence. RBL-2H3 cells were transfected by electroporation with the active construct or a negative control construct. Stably transfected clones were selected using 8 µg/mL Blasticidin (Invitrogen), and GFP protein expression was verified by green fluorescence.

4.3. RNA extraction and quantitative real-time RT-PCR

RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was made using SuperScript™ First-Strand (Invitrogen). SYBR GREEN PCR Master Mix (Applied Biosystems) was used in real-time PCR measurements using an ABI PRISM 7900HT Sequence Detection System. Beta actin message was measured as a housekeeping gene for normalization. Primers for real-time PCR were as follows: Primer A 5'-CATTGTGAGTGCCACCATTC (5' cDNA primer for rat *Fcer1a* (bp 288, GenBank Accession [M17153](#))); Primer B 5'-TTCTTCCAGCTACGGCATCT (3' complementary primer for Primer A (bp 461, product length 174 bp)). Primer C 5'-CCTGGCTCCTAGCACCATGA (5' rat beta actin cDNA primer (bp 1038, GenBank Accession [BC063166](#))); Primer D 5'-TCTGCTGGAAGGTGGACAGT (3' complementary matching for Primer C (bp 1144, product length 107 bp)).

4.4. Co-culture and IL-4 quantitation

For co-culture experiments a freshly thawed aliquot of *M. pneumoniae* or other agents was added to appropriate wells of a washed, pre-warmed 24 well culture plate containing RBL cells and transfected sublines in RPMI 1640 with 10% fetal bovine serum and 20 mM HEPES buffer (pH 7.0) but no antibiotics. In some experiments RBL subclones were isolated by limiting dilution, grown to confluence and their IL-4 responses to IgE stimulation and *M. pneumoniae* determined. Agents used included *M. pneumoniae* strain M129 (10⁸ CFU/mL final) (about 100:1 mycoplasma:RBL cell ratio, a concentration previously determined to be optimal), low-dose oligomeric rat myeloma IgE (IR162) (0.5 µg/mL final) [40], mouse anti-rat CD32 monoclonal antibody (Mab) (FcBlock™, BD Biosciences 550273, mouse IgG₁κ isotype, azide-free, low endotoxin), and phorbol 12-myristate 13-acetate (PMA, Sigma P8139)/ionomycin (Sigma IO634) (40 ng/mL/800ng/mL). Examination of the pre-incubation time required for CD32 showed that the inhibitory effect was maximal by 30 minutes (data not shown). The plate was then briefly centrifuged at 2000 RPM (850 x g) to place the bacteria in contact with the adherent mast cells before incubation in a 37° C, humidified, 5% CO₂ incubator. After 4 hours incubation, culture supernatants were collected, filtered using 0.2 µ spin filters to remove cell debris and stored at -80°C until assayed. IL-4 protein in the supernatants was quantitated by using a rat cytokine ELISA (BD Biosciences), according to the manufacturer's instructions.

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Figure 1.

IL-4 production by a series of RBL cell subclones in response to IgE stimulation correlates with the response to *M. pneumoniae*. The IL-4 concentration in 4 hour supernatants following stimulation with IgE (0.5 µg/ml) or *M. pneumoniae* was compared. For each subclone the responses tended to be similar ($r^2 = 0.82$, $p < 0.0001$).

Figure 2.

Inhibition of IL-4 production by anti-CD32 Mab (ng/ml) in response to stimulation with oligomeric IgE, *M. pneumoniae*, or PMA/ionomycin. Results are expressed as a percentage of the response in the absence of anti-CD32 Mab (mean $\pm$ SD for two experiments).

Figure 3.

Message reduction for FcεRI α chain in RBL sublines stably transfected with RNAi construct. Shown are the means ± SD of the ratios of Tc for each subline to the parent RBL line normalized using β actin (n = 4, each performed in quadruplicate). Asterixes indicate p < 0.05 compared to sham control.

Figure 4.

Decreased IL-4 production in RNAi transfected mast cells in response to oligomeric IgE. Shown is the IL-4 production in four RNAi transfected sublines and the Sham control (mean $\pm$ SD, n = 4, representative results from a set of three experiments). Asterixes indicate $p < 0.05$ compared to sham control.

Figure 5.

IL-4 production by transfected RBL cell lines induced by *M. pneumoniae* co-culture. Shown is the mean IL-4 $\pm$ SD for three experiments, each performed in duplicate. Asterixes indicate $p < 0.05$ compared to sham control.

Figure 6.

Model for *M. pneumoniae*-induced mast cell activation. Binding of diverse cell surface sialoglycoproteins by the bacterial adherence organelle clusters FcεRI on the mast cell surface in sufficient densities to initiate cellular activation. Unlike the situation with IgE-antigen-mediated activation, small changes in receptor density may abrogate the stimulatory effect by dilution of receptor number below a critical level.