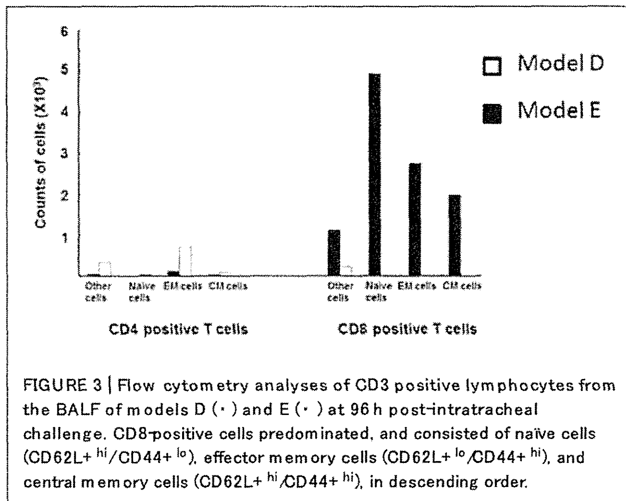

FIGURE 3 | Flow cytometry analyses of CD3 positive lymphocytes from the BALF of models D (·) and E (·) at 96 h post-intratracheal challenge. CD8-positive cells predominated, and consisted of naïve cells (CD62L+ ^{hi}/CD44+ ^{lo}), effector memory cells (CD62L+ ^{lo}/CD44+ ^{hi}), and central memory cells (CD62L+ ^{hi}/CD44+ ^{hi}), in descending order.

Humoral immunity

The humoral immune responses in Mp pneumonia were elucidated by the discovery of autoimmune-mediated phenomena involving cross-reactive antibodies to host organs. Neurologic manifestations following Mp infection can occur as a result of molecular mimicry by carbohydrate moieties of the abundant glycolipids in the Mp membrane and the lipoglycan capsule (Ang et al., 2002; Yuki, 2007). Autoimmune hematologic disorders can also occur following Mp infection— transient brisk hemolytic anemia, termed "paroxysmal cold hemogloblinuria." As for lung inflammation, how humoral immunity contributed to Mp pneumonia was unknown. However, patients with humoral deficiency seemed to become chronic carriers of Mp (Taylor-Robinson et al., 1980) or to undergo repeated episodes of Mp pneumonia (Roifman et al., 1986) or severe arthritis (Taylor-Robinson et al., 1978; Johnston et al., 1983), phenomena indicating that humoral immunity plays a role in protection against these organisms.

Cytokine profile in blood and BALF

Cytokines are important components of the lung defense mechanism and inflammation (Yang et al., 2004). Here we describe findings obtained from human patients and mouse models of Mp pneumonia.

Cytokines in BALF of human Mp pneumonia. A few studies have been reported concerning cytokine profiles in the BALF of human Mp pneumonia patients. Koh et al. reported that IL-4 levels and IL-4/IFN-γ ratios in BALF are significantly higher in children with Mp pneumonia than in patients with pneumococcal pneumonia or control participants (Koh et al., 2001). This suggests that a Th2-like cytokine response in Mp pneumonia is predominant, representing a favorable condition for IgE production. Yano et al. described an increased level of eosinophil cationic protein in BALF of all 10 Mp pneumonia patients studied, supporting the allergic aspects of Mp pneumonia (Yano et al., 2001).

Cytokine profile of BALF in Mp pneumonia mouse models. Previous reports of mice inoculated with live Mp described that Mp induced an increase in BALF of the concentrations of IL-17, KC, TNF-α, IL-6, IFN-γ, and IL-12 (Fonseca-Aten et al., 2005; Chu et al., 2006; Salvatore et al., 2007, 2008; Wu et al., 2007). Likewise, we demonstrated that our model E (Figure 1) mice had a significant increase in the levels of BALF cytokines, including IL-6, MCP-1, and RANTES, 24 h post-IT, when compared to those of model D mice (Figure 1) (Saraya et al., 2011), which was thought to be attributable to antecedent immunization with Mp antigen. Regarding the allergic aspect, Mp infection in airway epithelial cells can contribute to the pathogenesis of chronic asthma by inducing RANTES and tumor growth factor-β1 (Sohn et al., 2005). We also generated another mouse model (in which no adjuvant was used), as reported by Kurai et al. (2013a), in which mice were intraperitoneally immunized with only Mp antigen twice, on day −28 and day −21 (RI, repeated inoculation, group) or had no pretreatment (SI, single inoculation, group), followed by IT challenge with Mp antigen on day 0 for both groups (Figure 4). In this RI model, the levels of proinflammatory or Th2 cytokines in BALF, including IL-17, KC, IL-6, TNFα, and IL-4, were significantly higher than those of the SI model mice. Furthermore, immunohistochemical analysis of lung tissues collected on day 1 revealed IL-23 positive alveolar macrophages together with elevation of IL-17 both in the BALF and in the supernatants of lung-derived cells cultured with Mp antigen, which suggested activation of the IL-23/IL-17 axis (Iwakura and Ishigame, 2006). Likewise, Wu et al. reported that Mp infection of mouse lungs can be prolonged when IL-23 mediated IL-17 production is neutralized (Wu et al., 2007).

Cytokine profile of blood in human Mp pneumonia. Tanaka et al. reported that serum levels of IL-18 were elevated during the acute phase of Mp pneumonia (Tanaka et al., 2002), which suggested IL-18 and Th1 cytokines may play a significant role in the immunopathologic responses in Mp pneumonia. Conversely, other reports described polarization to Th2 in Mp pneumonia, because of increased levels of eosinophil cationic protein (63%, 17 of 27 cases) (Yano et al., 2001) or the detection of IgE antibody specific for Mp (Tipirneni et al., 1980; Yano et al., 1994; Seggev et al., 1996), indicating an allergic aspect of human Mp pneumonia. Esposito et al. reported that children with acute Mp infection and wheeze had higher IL-5 concentrations than did healthy controls (Esposito et al., 2002). Matsuda et al. reported that serum IFN-γ, IL-6, and IP-10 (Interferon γ-induced protein 10) levels were higher in patients infected with macrolide-resistant Mp genotypes than were those in patients infected with conventional Mp strains (Matsuda et al., 2013).

What are the key players leading to lung inflammation in Mp pneumonia?

We have postulated a process for the generation of human Mp pneumonia, which is described in Figure 5 and in the following sections.

Bronchial epithelial cells. Mp attaches to ciliated respiratory epithelial cells at the base of the cilia by means of a complex terminal organelle at one end of the elongated organism, which is mediated by interactive adhesins and accessory proteins clustered at the tip of the organelle. Briefly, Mp attaches to the bronchial

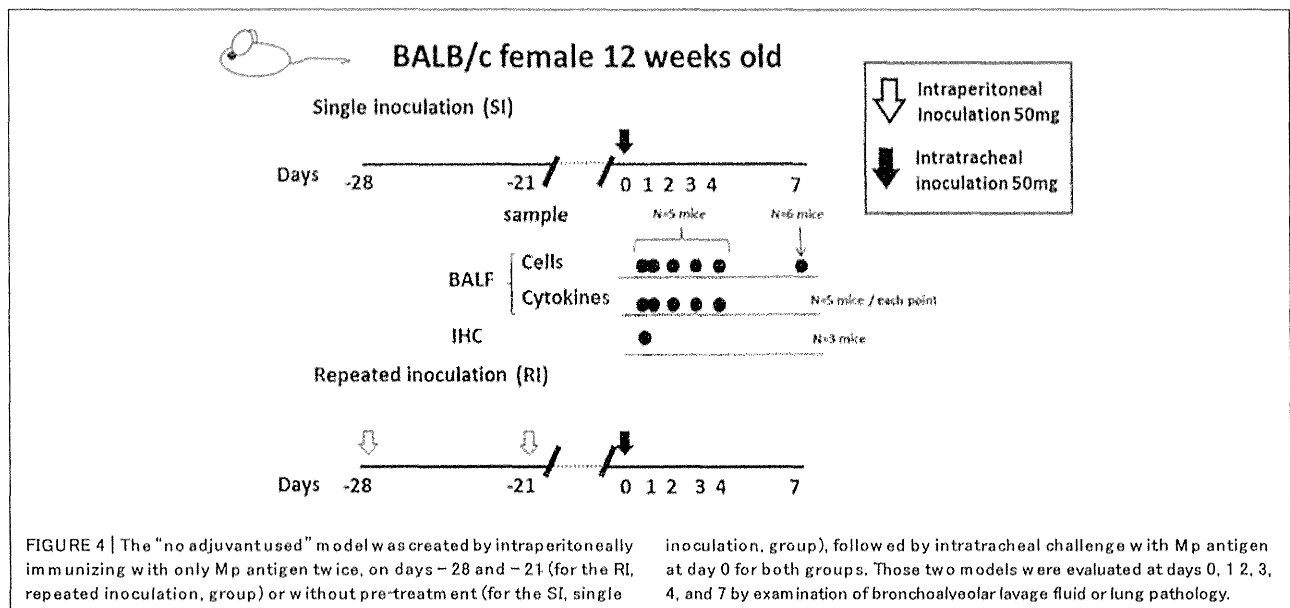

FIGURE 4 | The "no adjuvant used" model was created by intraperitoneally immunizing with only Mp antigen twice, on days − 28 and − 21 (for the RI, repeated inoculation, group) or without pre-treatment (for the SI, single inoculation, group), followed by intratracheal challenge with Mp antigen at day 0 for both groups. Those two models were evaluated at days 0, 1 2, 3, 4, and 7 by examination of bronchoalveolar lavage fluid or lung pathology.

epithelial cells via P1 adhesin (Razin and Jacobs, 1992), P30, and other structures (HMW1, HMW2, HMW4, HMW5, P90, and P65) (Waites and Talkington, 2004). Mp produces hydrogen peroxide and superoxide radicals, which induce oxidative stress in the respiratory epithelium. Dakhama et al. reported that Mp upregulated transforming growth factor (TGF)-β1 in primary cultures of normal human bronchial epithelial cells (NHBE), and RANTES in small airway epithelial cells (SAEC) (Dakhama et al., 2003), which would act in vivo together with increased IL-8 production on bronchial epithelial cells (Sohn et al., 2005).

Alveolar macrophages. First, Mp attaches to the bronchial epithelial cells. Next, macrophages, including AMs, would play a role as an innate host defense mechanism; however, to our knowledge there are no reports regarding the number of macrophages recruited or pre-existing in the bronchial lumen. AMs are the predominant macrophage type in the lung, constituting approximately 93% of the pulmonary macrophage population (Marriott and Dockrell, 2007). AMs originate from monocytes recruited from the blood, but replication of AMs makes a minor contribution to the total pool (Blusse Van Oud Alblas et al., 1981). In Mp pneumonia, it has been reported that TLR-2 signaling is involved in inflammatory cell activation by Mp-derived lipoproteins (Shimizu et al., 2008). Chu et al. demonstrated that expression of TLR-2 mRNA and protein on alveolar macrophages and the recruitment of adaptor protein MyD88 increase after Mp infection (Chu et al., 2005). AMs are early effectors of innate immunity against any bacteria, and Mp was recognized via TLR1, 2, and 6 on AMs. Previously, studies using our models of germ-free (Hayakawa et al., 2002) and other gnotobiotic mice (Sekine et al., 2009), as well as another study by Chu et al. using BALB/c and C57BL/6 mice (Chu et al., 2006), in turn demonstrated that pre-immunization with live Mp or Mp antigen significantly augmented inflammatory responses after the second challenge. Likewise, Saraya et al. showed enhanced expression of TLR-2 on bronchial epithelial cells and AMs after two immunizations with Mp antigens plus adjuvant alum (Figures 1E, 2E,F) (Saraya et al., 2011; Saraya, 2013). Based on those animal model studies, it is likely that subclinical, latent infection with Mp in the lower respiratory tracts may up-regulate TLR-2 expression on AMs and bronchial epithelial cells, which augments Mp reactivity.

AMs can also secrete proinflammatory cytokines (IL-6, TNF-α and IL-1β, IL-18, MIP-1α KC, RANTES, IL-12, IL-23, and MCP-1 (Saraya et al., 2011; Kurai et al., 2013a; Narita et al., 2000), which are associated with neutrophilic infiltration. Although the number of AMs after two immunizations (models D and E, Figures 1D,E) was equal, we demonstrated that the accumulation of abundant neutrophils in the alveolar spaces as early as 8 h post-IT in model E (Figure 1E) was attributable to the effect of antecedent immunization with Mp antigen, as compared with model D animals (Figure 1D) (Saraya et al., 2011). Vigorous recruitment of neutrophils is one of the most important components of the initial innate immune response (Craig et al., 2009). Immunohistochemical analysis at 8 h post-IT of Model E (Figure 1E) showed that AMs secreted RANTES, which is a known, potent chemoattractant for neutrophils or lymphocytes. However, abundant recruited neutrophils in the alveolar spaces did not produce RANTES (Figure 6A). Bronchial epithelial cells were also immunohistochemically stained with RANTES at 48 h post-IT (Figure 6B).

In this regard, our mouse models for Mp pneumonia (Figure 1E) indicated the possibility that even in humans, latent respiratory infection might trigger the inflammation or enhance the host defense through up-regulation of TLR-2 expression on bronchial epithelial cells and AMs, followed by production of IL-23-dependent IL-17 production (Wu et al., 2007; Kurai et al., 2013a) or other chemokines, including RANTES.

Lymphocytes. As mentioned above in the Section "Host defenses," for human Mp pneumonia, to our knowledge, no data are

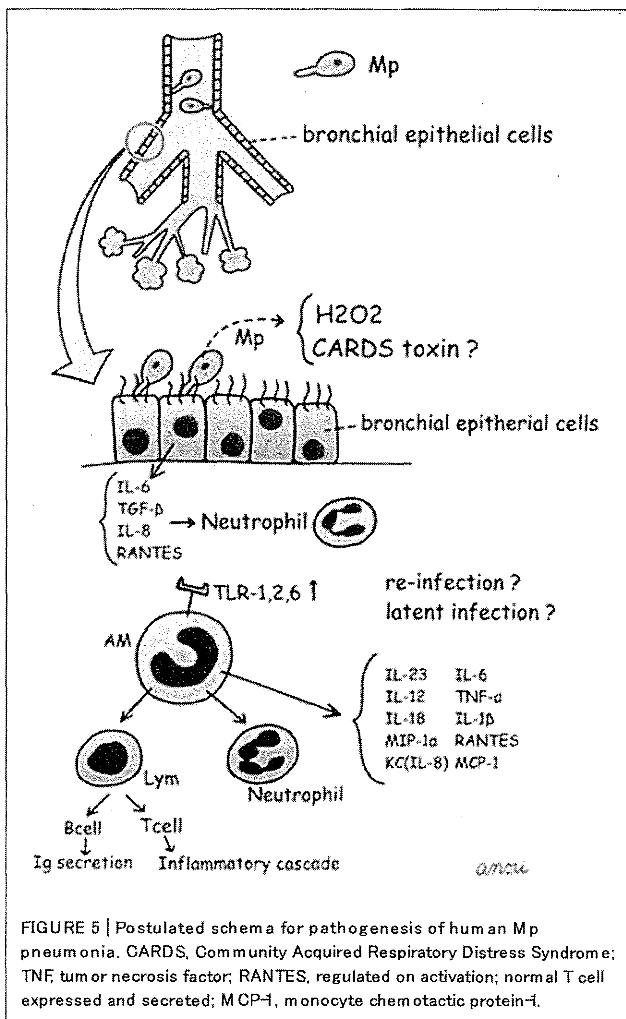

FIGURE 5 | Postulated schema for pathogenesis of human Mp pneumonia. CARDS, Community Acquired Respiratory Distress Syndrome; TNF, tumor necrosis factor; RANTES, regulated on activation; normal T cell expressed and secreted; MCP-1, monocyte chemotactic protein-1.

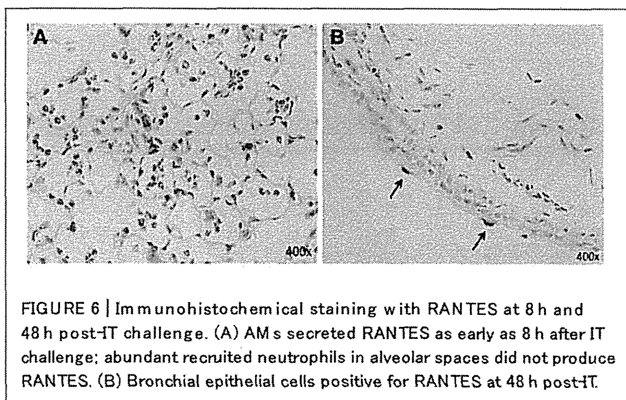

FIGURE 6 | Immunohistochemical staining with RANTES at 8 h and 48 h post-IT challenge. (A) AMs secreted RANTES as early as 8 h after IT challenge; abundant recruited neutrophils in alveolar spaces did not produce RANTES. (B) Bronchial epithelial cells positive for RANTES at 48 h post-IT.

available regarding whether the presence of lymphocytes in the lung or BALF is due to a specific reaction to Mp. Regarding memory T cells, no combination of chemokine receptors and/or adhesion molecules has apparently been identified to date that imparts a preferential migration to the bronchial compartment or alveolar compartment (Pabst and Tschernig, 1995; Wardlaw et al., 2005; Kohlmeier and Woodland, 2006). Lymphocytes constitute about 10% of all cells in the BALF of healthy adults. Less than 10% of the lymphocytes in the BALF are B cells, and among the T cells, CD4+ cells outnumber CD8+ cells (Pabst and Tschernig, 1997), with a CD4+ /CD8+ ratio of 1.7 (Pabst and Tschernig, 1995). There are more so-called "memory" (> 85%) than "naive" T lymphocytes in the BALF, which is different from the composition of lymphocytes in other lung compartments (Pabst and Tschernig, 1997). Studies using our mouse model E (Saraya et al., 2011) showed that CCL5 (also known as RANTES) was highly expressed in lung cells, including bronchial epithelial cells, AMs, and lymphocytes. RANTES is produced by activated T cells, fibroblasts, platelets, kidney epithelial cells, macrophages, and endothelial cells, and is chemotactic for memory T cells, monocytes, and eosinophils (Schall et al., 1990; Alam et al., 1993; Monti et al., 1996) as well as neutrophils (Pan et al., 2000), triggering its receptor, CCR5 (Charo and Ransohoff, 2006). Use of our model E demonstrated CCR5-positive lymphocytes in the PBVA, implicating the contribution of RANTES in lung inflammation. Thus, as mentioned in the "Host defenses" Section above, various proinflammatory cytokines and C-C chemokines (RANTES, MCP-1) (Gunn et al., 1997; Johnston et al., 1999) might be key players in the development of Mp pneumonia, both in the acute and chronic phases (Conti and Digioacchino, 2001). Of note, lung pathology seemed to differ according to host characteristics (Th1, Th2, and Th17) which might be a non-specific reaction to Mp.

CLINICAL FEATURES

GENERAL ASPECTS

Mp infection is usually self-limited and rarely fatal. Mp infection causes both upper and lower respiratory infections, and pneumonia occurs in 3-13% of infected persons (Clyde, 1993). Clinical features of Mp infection vary among different ages, in that patients under 2 years of age tend to have upper respiratory infections, while 6-19-year-olds tend to have pneumonia (Foy et al., 1966; Denny et al., 1971). Two major subtypes of the P1 gene are known to occur in Mp, and this subtype shift phenomenon may have a relation to Mp pneumonia outbreaks (Kenri et al., 2008). The severity of Mp pneumonia seems to depend on the Mp bacterial load rather than Mp subtype (Nilsson et al., 2010). The incubation period for Mp infection is about 2-4 weeks, and characteristic findings of adult Mp pneumonia are younger age, fewer comorbid diseases, shorter length of stay in hospital, and lower mortality than any other group of CAP patients. Prospective studies of patients with Mp pneumonia from Germany (Von Baum et al., 2009) and Japan (Goto, 2011) revealed average (mean ± SD) ages of 41 ± 16 and 37.7 ± 16.6, respectively.

Severity scores are widely used for assessing the requirement for admission or when describing mortality rates, including the pneumonia severity index (PSI) or CURB-65 (Cilloniz et al., 2011). Gradual onset of respiratory or constitutional symptoms such as cough, fever, headache, and malaise are relatively common symptoms in Mp pneumonia. In particular, dry cough was usually observed in patients during early-phase Mp pneumonia, but it persists for a long period as a typical symptom. Goto (2011) reported that the mean body temperature in adult

Japanese patients with Mp pneumonia was 37.7 ± 1.0°C and that 29.2% of patients had a temperature no greater than 37.0°C. Analysis of physical examination data revealed that more than half of patients with Mp pneumonia had no audible crackles and were likely to have late-inspiratory crackles as compared with those infected with typical pathogens (Norisue et al., 2008). On laboratory examination, Mp pneumonia patients had relatively lower leukocyte counts than did those having pneumonia from other causes (Von Baum et al., 2009).

Macrolide was not the preferable treatment for S. pneumoniae pneumonia, as opposed to pneumonia from atypical pathogens, including Mp because highly macrolide-resistant *Streptococcus pneumoniae* was emerging to become dominant in Japan (Goto et al., 2009). The Japanese Respiratory Society (JRS) recommended discrimination of atypical pneumonias from CAP due to other pathogens (Committee For The Japanese Respiratory Society Guidelines For The Management Of Respiratory, 2006), and proposed six characteristic signs and symptoms of Mp pneumonia that can easily discriminate the two. Indeed, Yin et al. confirmed that use of these criteria has high sensitivity (88.7%) and specificity (77.5%) (Yin et al., 2012) for the diagnosis of Mp pneumonia if four or more of the proposed factors are present. The six factors are as follows: (i) < 60 years of age; (ii) absence of, or only minor, underlying diseases; (iii) stubborn cough; (iv) adverse findings on chest auscultation; (v) absence of sputum or identifiable etiological agent by rapid diagnostic testing; and (vi) a peripheral white blood cell count < 10,000/μL.

SPECIAL CIRCUMSTANCES

Latent respiratory infection/asymptomatic carrier

Mp pneumonia is a one of the leading causes of CAP, and it may exacerbate symptoms of underlying asthma (Nisar et al., 2007), especially in up to 25% of children with wheezing (Henderson et al., 1979); it was identified in 20% of exacerbations in asthmatic children requiring hospitalization and in 50% of children experiencing their first asthmatic attack (Biscardi et al., 2004). Spuesens et al. demonstrated that Mp was carried at high rates in the upper respiratory tracts of healthy children (Spuesens et al., 2013). However, Cunningham et al. could not confirm the relationship between asthma symptoms and Mp infection in children aged 9-11 years (Cunningham et al., 1998). Another study showed that most Mp patients, positive by PCR, had respiratory symptoms; that Mp DNA might be detected several months after acute infection; and that asymptomatic carriage of Mp is uncommon even after the outbreak period (Nilsson et al., 2008).

Especially for adults, to our knowledge, there have been few reports regarding the frequency of latent respiratory infection with Mp. Wadowsky et al. reported that tests of 473 respiratory specimens by culture, PCR, or both identified only four episodes (0.8%) of Mp-associated illness in adolescents and adults (n = 491) with persistent cough (Wadowsky et al., 2002). Thus, the frequency of the Mp carrier state or the bacterial load might be different between children and adults, or between healthy and asthmatic individuals. Indeed, our epidemiological data throughout the year demonstrated that among admitted adult patients with diverse respiratory diseases, including acute exacerbation of idiopathic interstitial pneumonia (n = 15), pneumonia (n = 43), asthma attack (n = 14), and exacerbation of COPD (n = 11), there were 4 cases of definite Mp pneumonia as diagnosed by a CF antibody titer increased ≥ 4-fold or passive particle agglutination test ≥ 640, but with no identifiable Mp in the throat/nasopharynx or sputum by both culture and PCR methods (Kurai et al., 2013b) (Figure 7). This might reflect the fact that Mp acted only to trigger the lower respiratory symptoms or pneumonia, but the bacterial load was low, resulting in a latent respiratory infection or even in Mp pneumonia, especially in adult patients.

Macrolide-resistant Mp pneumonia

Macrolide-resistant Mp emerged and was widespread in East Asia after 2000. The reasons for the regional differences in macrolide-resistant Mp have not been elucidated. The A2063G mutation has been found to be most prevalent in macrolide resistant Mp isolates, followed by the A2064G mutation; these mutations are associated with increased minimum inhibitory concentrations to macrolides, including erythromycin, azithromycin, and clarithromycin.

Previous studies revealed that macrolide-resistant Mp pneumonia patients had a prolonged fever compared to those with macrolide-susceptible Mp pneumonia, in both children and adults (Suzuki et al., 2006; Cao et al., 2010; Pereyre et al., 2012; Yoo et al., 2012). In patients with macrolide-resistant Mp pneumonia, clinical findings, including symptoms, laboratory results, radiology, the complication of respiratory failure, and mortality were not different from those of patients with macrolide-susceptible Mp pneumonia. However, persistent fever over 48 h after initiation of macrolide may point to the presence of macrolide-resistant Mp (Miyashita et al., 2013).

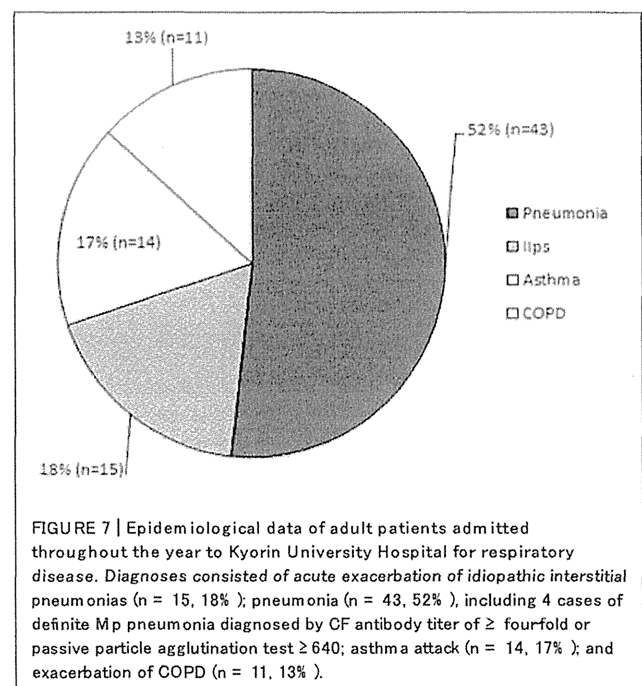

FIGURE 7 | Epidemiological data of adult patients admitted throughout the year to Kyorin University Hospital for respiratory disease. Diagnoses consisted of acute exacerbation of idiopathic interstitial pneumonias (n = 15, 18%); pneumonia (n = 43, 52%), including 4 cases of definite Mp pneumonia diagnosed by CF antibody titer of ≥ fourfold or passive particle agglutination test ≥ 640; asthma attack (n = 14, 17%); and exacerbation of COPD (n = 11, 13%).

Fulminant Mp pneumonia

Mp pneumonia is usually mild and rarely fatal. The severity of Mp pneumonia seems to depend on the Mp bacterial load rather than the Mp genotype (Nilsson et al., 2010). Among patients with Mp pneumonia, 3-4% develop severe, life-threatening illness with respiratory failure and acute respiratory distress syndrome (Holt et al., 1977; Fraley et al., 1979; Koletsky and Weinstein, 1980; Chan and Welsh, 1995; Ito et al., 1995; Takiguchi et al., 2001; Tsuruta et al., 2002; Miyashita et al., 2007). Two groups (Chan and Welsh, 1995; Miyashita et al., 2007) reported that the delayed administration of adequate antimicrobials was noted in severe Mp pneumonia patients, at an average of 9.3 or 15 days, respectively, which may be the most important reason for the development of fatal respiratory failure. However, some cases who had adequate antimicrobials within 3 days after the onset of the disease progressed to respiratory failure (Miyashita et al., 2007). Izumikawa et al. reviewed 52 Japanese cases of fulminant Mp pneumonia (Izumikawa et al., 2014), which was defined as the presence of Mp pneumonia with hypoxia, and reported that no apparent risk factors for fulminant Mp pneumonia were identified, but concluded that initial inappropriate use of antimicrobials may be a risk factor.

RADIOLOGICAL FEATURES

A wide spectrum of findings on thin-section CT have been reported, such as ground glass opacities (GGO), consolidation, bronchial wall thickening, centrilobular nodules, interlobular septal thickening, pleural effusion, mosaic attenuation, air trapping, and lymphadenopathy (Kim et al., 2000; Reittner et al., 2000; Chiu et al., 2006; Lee et al., 2006; Miyashita et al., 2009). Each of those radiological findings are non-specific, but Miyashita et al. reported that bronchial wall thickening and centrilobular nodules on thoracic CT would be a clue to the diagnosis (Miyashita et al., 2009). Figure 8 shows typical HRCT findings such as consolidation with air bronchograms surrounded by a crazy paving appearance (A), consolidation with reticular shadow (B), consolidation with GGO (C), GGO with interlobular septal thickening (D), crazy paving appearance (E), bronchial wall thickening with centrilobular nodules (F), diffuse centrilobular nodules.

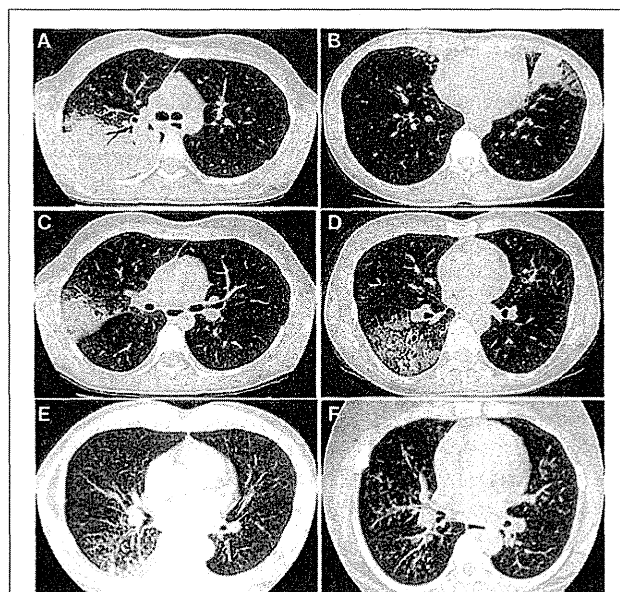

FIGURE 8 | The HRCT findings of Mp pneumonia are characterized as (A) consolidation with air bronchogram surrounded by a crazy paving appearance, (B) consolidation with reticular shadow, (C) consolidation with GGO, (D) GGO with interlobular septal thickening, crazy paving appearance, (E) bronchial wall thickening with centrilobular nodules, (F) diffuse centrilobular nodules.

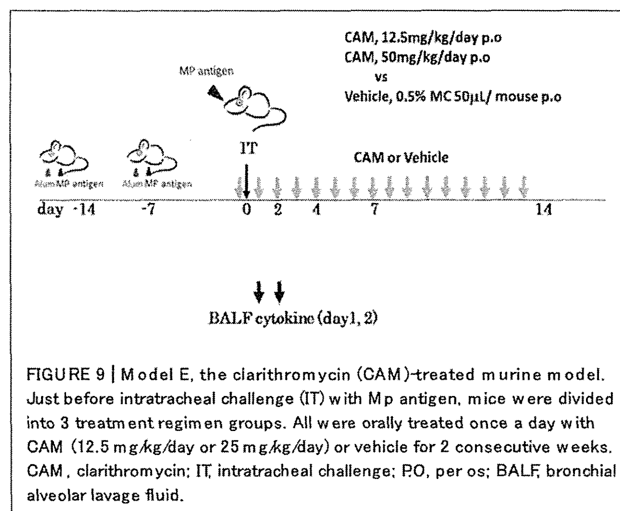

FIGURE 9 | Model E, the clarithromycin (CAM)-treated murine model. Just before intratracheal challenge (IT) with Mp antigen, mice were divided into 3 treatment regimen groups. All were orally treated once a day with CAM (12.5 mg/kg/day or 25 mg/kg/day) or vehicle for 2 consecutive weeks. CAM, clarithromycin; IT, intratracheal challenge; P.O, per os; BALF, bronchial alveolar lavage fluid.

DIAGNOSTIC METHODS

CULTURE

Culture is the "gold standard" method for diagnosis of Mp infection and is essential for further analysis, including drug resistance tests, although it is useless as a rapid diagnostic method because of the low sensitivity and the need for incubation for several weeks in specialized culture medium.

SEROLOGICAL METHODS

There are many diagnostic serological tests, although these serological tests and their interpretations are not standardized. Serological methods, such as complement fixation (CF), passive agglutination (PA), and detection of IgG and IgM by enzyme immunoassays (EIA) were conventionally used for diagnosis of Mp infection. CF tests measure IgM and IgG antibodies together, but these antibodies are non-specific. The target for PA tests was mainly IgM antibody, but seemed to be less specific for Mp than the Mp-specific IgM enzyme-linked immunosorbent assays (ELISA) (Barker et al., 1990).

Paired sera for CF, PA and Mp-specific IgG EIA tests are widely used for epidemiological studies and are regarded as a standard method for diagnosis. The definition of Mp infection was based on the serological finding of a four-fold titer rise (in CF or PA tests), and seroconversion or a significant increase, of Mp IgG during the convalescent phase compared with the acute phase. Single high titers were also considered markers of Mp infection,

and the difference of cut-off titer used in various studies has a great impact on the resulting epidemiological data. If either CF titers are higher than 1:64 or 1:128, or PA titers are higher than 1:320 or 1:640, a diagnosis of Mp infection was made (Marston et al., 1997; Dorigo-Zetsma et al., 1999; Templeton et al., 2003; Beersma et al., 2005; Kim et al., 2007). Measurement of Mp-specific IgM antibodies by EIA has been commercially available for the diagnosis of Mp infection during the early phase. Beersma et al. (2005) reported that twelve IgM EIA assays showed various diagnostic yields when compared to PCR-proved Mp pneumonia as the reference standard. The sensitivity and specificity of these IgM EIA assays were 35-77% and 49-100%, respectively, and those assays had low diagnostic yields within a week after initial onset. Mp-specific IgM (EIA) assays were less useful for adults with autoantibodies or other infectious diseases, such as Epstein-Barr virus, *Streptococcus pyogenes* and *Treponema pallidum*, because of the tendency of these to produce false positives (Petitjean et al., 2002; Beersma et al., 2005).

NUCLEIC ACID AMPLIFICATION METHODS

Polymerase chain reaction (PCR)-based methods using respiratory samples have been developed for rapid Mp diagnosis. This application was limited to select hospitals because complicated procedures and expensive systems are required. Diagnosis of Mp infection using PCR was inconsistent among individual studies because of many factors, as follows: patients' ages; intervals between onset of symptoms and sampling specimens; types of specimen sampling methods; target lesion of PCR; and technical procedures (Raty et al., 2005; Loens et al., 2009; Thurman et al., 2009). He et al. showed that PCR-based diagnosis was superior to IgM-based diagnosis in Mp-infected patients less than 3 years of age; an immature immune response to Mp may explain this discrepancy (He et al., 2013). A meta-analysis of PCR-based diagnosis for Mp infection showed that sensitivity and specificity were 0.62 (95% CI, 0.45-0.76) and 0.96 (95% CI, 0.93-0.98), respectively (Zhang et al., 2011).

As for Mp pneumonia, PCR and serological diagnosis had good concordance in adult patients; PCR-based diagnosis had lower sensitivity (66.7%) compared to serological diagnosis as the reference standard. This result was consistent with those in other reports on Mp CAP in adults (Pitcher et al., 2006; Martinez et al., 2008; Qu et al., 2013). The sensitivity and specificity of PCR-based diagnosis in these studies were 40.7-66.7% and 88.8-98.5%, respectively; the reference standard was a serological diagnosis (Table 3).

Loens et al. and Raty et al. described that if a sputum sample is available, it might be better for Mp detection in patients with Mp pneumonia than nasopharyngeal or oropharyngeal swabs (Raty et al., 2005; Loens et al., 2009). A nucleic acid amplification method, termed loop-mediated isothermal amplification (LAMP), was introduced in order to improve the complicated system of PCR, and LAMP results were concordant with PCR results (Saito et al., 2005).

In the early phase of the illness, the preferred diagnostic methods seemed to be culture and nucleic acid amplification. In the late phase, those methods are useless because of the low Mp load in the airways; furthermore, regarding the limited value of single serum samples, paired serological examinations would be required for diagnosis (Thurman et al., 2009). In conclusion, no reliable simple method exists for accurate diagnosis; therefore, we recommend the culture and nucleic acid amplification in the early phase, and serological examinations in the late phase, respectively, especially in the patients with severe pneumonia and/or who satisfied four or more of the proposed factors as described in "General aspects."

Table 3 | Comparison of diagnostic methods.

	Sensitivity (%)	Specificity (%)	Comment
Culture	55.6	94.9	Isolation of Mp is slow and insensitive, and therefore is not recommended for routine use.
PCR	40.7-66.7	88.8-98.5	Rapid diagnosis is possible, but is costly and complicated procedures are needed. Therefore PCR-based diagnosis is limited to a few laboratories.
Serology IgM	7.4-77	49-100	Diagnostic yields for Mp IgM tests were variable, according to available assays. Use of paired sera for CF, PA or IgG analysis is preferable.

(Pitcher et al., 2006; Martinez et al., 2008; Qu et al., 2013). Most of data are from lower respiratory infections in adults, including pneumonia patients.

EXTRAPULMONARY MANIFESTATIONS

Although direct invasion, neurotoxin production, or an immune-mediated process have been proposed, the mechanisms underlying extrapulmonary manifestations of Mp infection remain largely unknown. These are diverse (Foy et al., 1983; Lind, 1983; Narita, 2010) and include central nervous system diseases such as encephalitis, aseptic meningitis, polyradiculitis, cerebellar ataxia, and myelitis (Guleria et al., 2005; Tsiodras et al., 2006); cardiovascular diseases such as pericarditis, endocarditis, and myocarditis; the dermatological diseases Stevens-Johnson syndrome, erythema multiforme (Cherry, 1993; Lamoreux et al., 2006), erythema nodosum, anaphylactoid purpura, and acute urticaria (Kano et al., 2007); hematological diseases including autoimmune hemolytic anemia (cold agglutinin disease), hemophagocytic syndrome, disseminated intravascular coagulation, and thrombocytopenic purpura (Cassell and Cole, 1981); inflammatory diseases including conjunctivitis, iritis

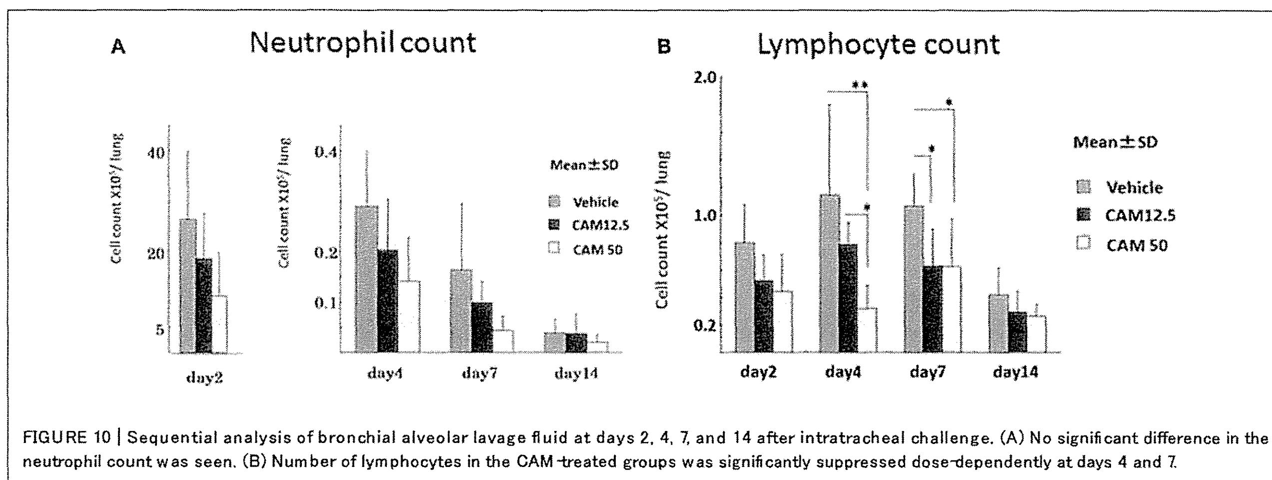

FIGURE 10 | Sequential analysis of bronchial alveolar lavage fluid at days 2, 4, 7, and 14 after intratracheal challenge. (A) No significant difference in the neutrophil count was seen. (B) Number of lymphocytes in the CAM-treated groups was significantly suppressed dose-dependently at days 4 and 7.

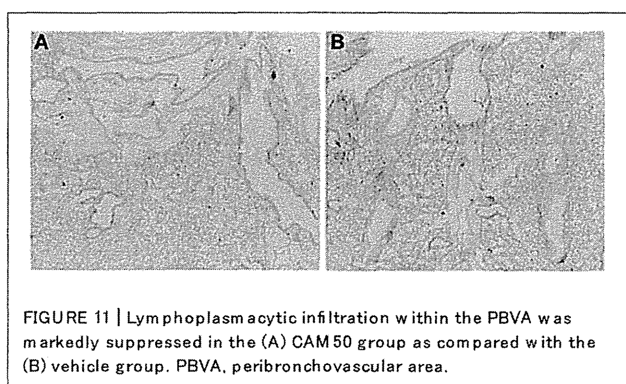

FIGURE 11 | Lymphoplasmacytic infiltration within the PBVA was markedly suppressed in the (A) CAM50 group as compared with the (B) vehicle group. PBVA, peribronchovascular area.

(Salzman et al., 1992), uveitis (Weinstein et al., 2006), and arthritis (Franz et al., 1997); and otitis media. The presence of these extrapulmonary manifestations is itself evidence of human immune system interaction with Mp.

TREATMENT

The recommended therapy for microbiologically confirmed Mp pneumonia is use of macrolides (CAM: clarithromycin and AZM: azithromycin) or tetracyclines, and fluoroquinolones are an alternative choice (Lim et al., 2009). However, neither tetracyclines nor fluoroquinolones are recommended for young children under 8 years of age because of their adverse effects, such as permanent yellowing or graying of the teeth, and abnormalities of articular cartilage and the QT interval. Therefore, macrolide-resistant Mp pneumonia is a major concern for children who require treatment. Several studies showed that macrolide-resistant Mp was susceptible to tetracycline and fluoroquinolone in vitro (Eshaghi et al., 2013; Hong et al., 2013). Minocycline or doxycycline, both tetracyclines, quickly decreased the loads of macrolide-resistant Mp and were effective against the resistant pathogen in humans. Okada et al. showed that tosufloxacin, a fluoroquinolone, seemed to be inferior to minocycline or doxycycline in clinical use (Okada et al., 2012). However, macrolides have an immunomodulatory or bacteriological effects even on a mouse model with macrolide-resistant Mp strain (Kurata et al., 2010). Therefore, even in the area of high resistant to macrolides such as Japan, JRS recommend the use of macrolides as first therapy for Mp pneumonia together with the use of method for differential diagnosis of atypical pneumonia and bacterial pneumonia.

Infectious Diseases Society of America (IDSA) and the American Thoracic Society (ATS) joint guidelines on adult CAP described that patients with CAP should be treated for a minimum of 5 days (level I evidence), and most patient become clinically stable within 3-7 days, so longer durations of therapy are rarely necessary (Mandell et al., 2007), but JRS guidelines does not refer to the optimal duration of the treatment. Smith CB et al. showed that tetracycline and erythromycin improve symptoms in adult volunteers who experimentally infected with Mp, but recurrence of Mp pneumonia was noted after completion of 7 days treatment with tetracycline (Smith et al., 1967).

Thus, the optimal antimicrobial dosage and duration are not clear; however, 10-14 days of therapy is generally recommended. Effective treatment of Mp pneumonia shortens the duration of fever and might prevent aggravation (Denny et al., 1971; Izumikawa et al., 2014).

IMMUNOMODULATIVE EFFECTS OF MACROLIDE THERAPY

Macrolides have direct effects on neutrophil function and production of cytokines involved in inflammation cascades (Zarogoulidis et al., 2012).

For Mp infections, 14- or 15-membered ring macrolides usually are considered the first-line agents, which are well known for anti-inflammatory, immunomodulative effects (Wales and Woodhead, 1999). CAM is a macrolide with a 14-atom lactone ring, and attenuation of inflammatory responses has been reported in both animal models of Mp pneumonia (Kurata et al., 2010) and in humans with respiratory diseases (Kudoh et al., 1998).

To examine the immunomodulative effects of CAM, mice in model E (Figure 1E) were treated with three different regimens, as follows: (Figure 9) orally with CAM at two doses (CAM12.5 group: 12.5 mg/kg/day or CAM50 group: 50 mg/kg/day); or with vehicle (methylcellulose), all at 1.5 h just before IT with Mp

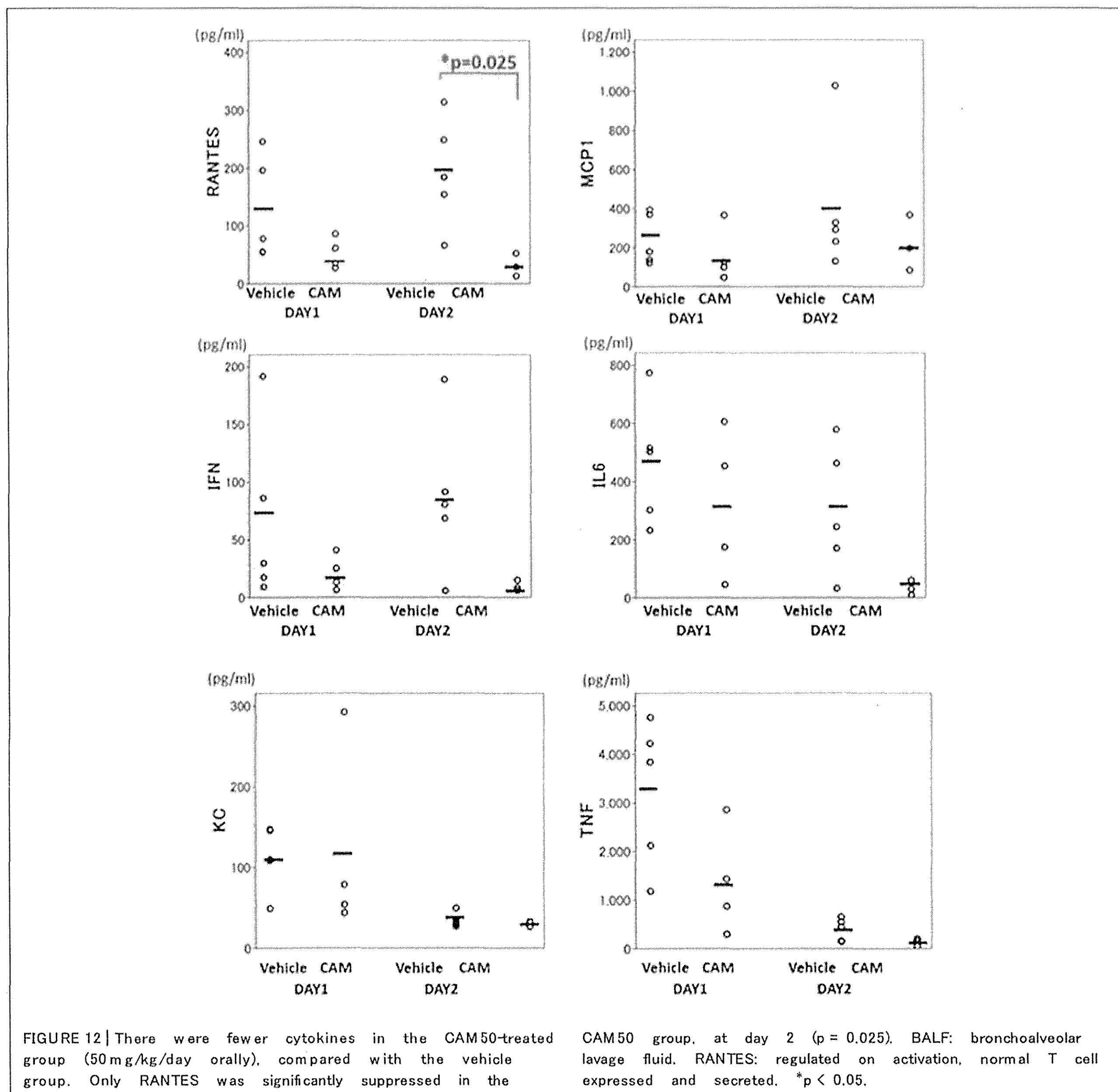

FIGURE 12 | There were fewer cytokines in the CAM50-treated group (50 mg/kg/day orally), compared with the vehicle group. Only RANTES was significantly suppressed in the CAM50 group, at day 2 ($p = 0.025$). BALF: bronchoalveolar lavage fluid. RANTES: regulated on activation, normal T cell expressed and secreted. $^{*}p < 0.05$.

antigen (day 0) (Saraya et al., 2007a; Saraya and Goto, 2008). Just before and after IT, the 3 groups were orally treated once a day with CAM or vehicle for 2 consecutive weeks. On BAL cell differential count analysis, there were no significant differences in the neutrophil count among the 3 groups in any phase (Figure 10A). However, the number of lymphocytes in the CAM-treated groups was significantly suppressed in a dose-dependent manner at day 4, and the effect was still recognized at day 7 (Figure 10B). Pathological assessment at day 4 post-IT revealed that the lymphoplasmacytic infiltration within the PBVA was markedly suppressed in the CAM50 group (Figure 11A), as compared with that of the vehicle group (Figure 11B).

BALF cytokines in the CAM50 group seemed to be lower than those of the vehicle group, and only RANTES was significantly suppressed in the former group, at day 2 ($p = 0.025$) (Figure 12). Those data suggested that oral administration of CAM has immunomodulative effects on lung inflammation even in the early phase of Mp pneumonia. This dose-dependent immunomodulative effect of CAM was consistent with previously reported results of a study using an experimental Mp pneumonia mouse model (Tagliabue et al., 2011).

STEROIDS AS ADDITIVE THERAPY

Animal experimental models (Tagliabue et al., 2008; Hirao et al., 2011) showed that corticosteroids down-regulate the host

immune response. Furthermore, treatment with the combination of clarithromycin and a corticosteroid, compared to clarithromycin alone, resulted in a significantly greater reduction of IL-12 p40 and RANTES (Tagliabue et al., 2008). Izumikawa et al. (2014) reported that a majority of human patients with fulminant Mp pneumonia had improved respiratory function on steroid treatment within 3-5 days, which was considered to be an effect of suppressing hyperactivated cellular immunity. Radisic et al. reported on the suppressing effects of steroids on the cell-mediated immune response (Radisic et al., 2000), and that acute respiratory distress syndrome (ARDS) secondary to Mp infection is a lymphoid cellularity ARDS caused by a harmful, "over-reacting" cell-mediated immune response, which could potentially be tapered by the use of steroids. Thus, steroid use would be the preferable treatment of patients with fulminant Mp pneumonia in light of the immune response.

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Congenital Anomalies

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CASE REPORT

Prenatal molecular diagnosis of X-linked hydrocephalus via a silent C924T mutation in the *L1CAM* gene

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ABSTRACT **We present a case of a patient whose *L1CAM* gene in X-chromosome has a C924T transition. Her first son's ventriculomegaly was prenatally detected. A mature infant was born, his head circumference was large, and thumbs were bilaterally adducted. X-linked hydrocephalus (XLH) was suspected. The DNA examination revealed that both her and boy's *L1CAM* gene had a C924T transition. She became pregnant 5 years later and amniocentesis was performed. The results of cytogenetic analysis revealed that the fetus was female. She continued her pregnancy and delivered a healthy girl. She again became pregnant 3 years later. The chromosomal analysis revealed that the fetus was male. Fetal DNA analysis determined that the fetus had the inherited mutation. She chose to terminate the pregnancy. A C924T mutation can be disease causing for XLH, and the detection of this mutation would aid in genetic counseling for the prenatal diagnosis of XLH.**

Key Words: *L1CAM* gene, prenatal diagnosis, silent mutation, X-linked hydrocephalus

INTRODUCTION

X-linked hydrocephalus (XLH) is one of the genetic forms of hydrocephalus. Mutations in the *L1CAM* gene are now known to be responsible for many cases of XLH. The *L1CAM* gene is located near the telomere of the long arm of the X chromosome and includes 28 exons. L1CAM is a neuronal cell adhesion molecule with important functions in the development of the nervous system. XLH has an incidence of 1/30 000 male births and is characterized by intellectual disability, spastic paraplegia, adducted thumbs, and agenesis of the corpus callosum and/or corticospinal tract (Fernández et al. 2012).

There are no hot mutation spots of *L1CAM* gene. To date more than 200 different mutations have been reported (Vos and Hofstra 2010). These mutations include frameshifts, missense mutations, stop codons, and alterations in splice site junction. A C924T transition is a silent mutation and that was thought to have no effect on the protein sequence. Recently it was reported that this mutation could cause alternative mRNA splicing and congenital hydrocephalus could occur. We present a case of a patient whose *L1CAM* gene has a C924T transition.

CLINICAL REPORT

A 25-year-old primigravida Japanese woman underwent prenatal management and care at a hospital near her residence. Fetal ventriculomegaly was detected during the 22nd weeks of gestation, so the patient was referred to the obstetrical outpatient clinic of the Niigata University Medical and Dental Hospital for closer examination. The patient had no other known complications or known genetic disease and had conceived spontaneously. Her family history was unremarkable. Her husband was 31 years of age and healthy. She and her husband were not consanguineous.

Fetal biparietal diameter was 60.8 mm and within normal range. A T2-weighted magnetic resonance imaging (MRI) examination revealed severe hydrocephalus and a thinning of the remaining cortex (Fig. 1). Amniocentesis for fetal chromosomal abnormality analysis was performed at the 25th week of gestation. The results of a cytogenetic analysis of cultured amniotic cells revealed a normal karyotype of 46, XY.

Serial ultrasonographic examinations were performed during the pregnancy. As the fetal biparietal diameter gradually enlarged, the patient was hospitalized. She underwent an elective cesarean section at the 38th week of gestation due to the cephalopelvic disproportion. A male infant weighing 2822 g was delivered with an Apgar score of 8 and 9 at 1 and 5 min post-delivery, respectively. His head circumference was 43.5 cm and his thumbs were bilaterally adducted. He received a ventriculoperitoneal shunt at 9 days of age. He had cerebral palsy and subsequently suffered from severe mental retardation.

When the first boy was 3 years old, his head MRI showed hypogenesis of the corpus callosum, thalamus concrescence, and a rippled ventricular wall after shunt placement. The boy's parents were justifiably concerned about the XLH being an inherited disease and asked for a genetic assessment. *L1CAM* gene testing was approved by the Ethics Committees of both the Niigata University and Osaka National Hospitals. The screening was carried out in accordance with the principles of the Declaration of Helsinki, as well as the Ethical Guidelines for Human Genome/Gene Analysis Research required by the Ministry of Education, Culture, Science, and Technology; the Ministry of Health, Labor, and Welfare; and the Ministry of Economy, Trade and Industry of Japan, and by the Guidelines for Genetic Testing in 2003 composed by the genetic medicine-related societies in Japan. After undergoing

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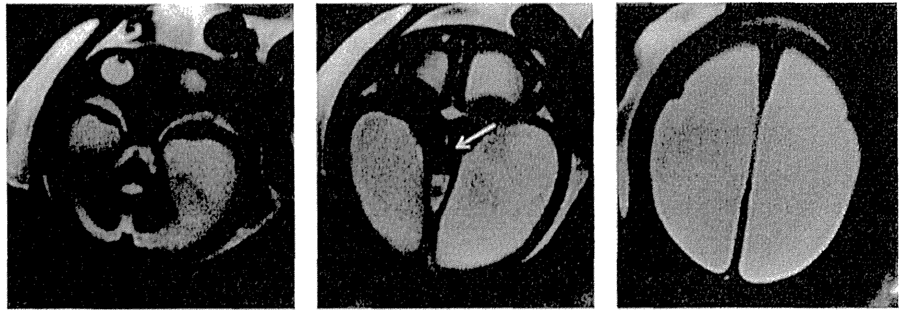

Fig. 1 Fetal magnetic resonance imaging (MRI) at 26th week of gestation. These figures were examined by T2-weighted MRI scan. TR/TE time was 11.90/95 m seconds. It demonstrated dilatation of the lateral ventricles and the third ventricle with aqueductal stenosis. An arrow indicates the aqueductal stenosis.

genetic counseling and having given their informed consent, the parents decided to undertake *L1CAM* gene analysis for their son and themselves. The DNA sequence by the Sanger method (Yamasaki et al. 2011) revealed that the boy's *LICAM* gene had a C924T transition in exon 8. His mother was X-chromosome heterozygous for the same mutation so that she could be a healthy carrier. However, this transition site was initially thought to have no effect on the protein sequence as the alteration affected the third base of a codon (G308G).

The mother and her husband desired to conceive their next baby 4 years after the birth of their first child and requested prenatal genetic testing as part of their genetic counseling. The Niigata University Ethics Committee approved a fetal sex determination by conventional karyotyping for prenatal diagnosis of XLH. We informed the couple that the sex determination could not definitely diagnose their next baby regarding XLH, and that there would be a 50% risk of XLH if the fetus was male and a 50% risk of a healthy carrier if the fetus was female. The woman became pregnant one year later and amniocentesis was performed at the 16th week of gestation. The results of cytogenetic analysis of cultured amniotic cells revealed that the fetus was female (46, XX). She continued her pregnancy and delivered a healthy girl at the 39th week of gestation.

Three years later, she again became pregnant, and requested prenatal genetic testing. Another Japanese family with familial XHL that carried the C924T mutation had been reported (Yamasaki et al. 2011). The Niigata University ethics committee approved a provisional karyotyping analysis for sex determination, and a mutation analysis for the C924T mutation if the fetus was male. At the 16th week of gestation, we obtained amniotic cells by amniocentesis. Fluorescence *in situ* hybridization testing showed that the fetus was in fact male. At the 17th week of gestation, fetal DNA was obtained from amniotic cells and DNA analysis determined that the fetus had inherited the C924T gene mutation. A conventional karyotyping test showed 46, XY. Atrial width was 5.9 mm and no other morphological findings could be detected by ultrasound examination. The mother and her husband chose to terminate the pregnancy at the 18th week of gestation. A stillborn male infant was delivered with a body weight of 240 g and with adducted thumb position. A pedigree of this family is shown in Figure 2.

DISCUSSION

XLH has been linked to the genetic L1 syndrome, which encompasses a wide spectrum of diseases, XLH being the most severe phenotype detected *in utero* (Adle-Biassette et al. 2013). The L1 syndrome results from mutations in the *L1CAM* gene located at Xq28 (Yamasaki et al. 1995). *L1CAM* is composed of 28 exons encoding the L1 neural cell adhesion protein, a molecule critical for nervous and enteric system development (Rosenthal et al. 1992).

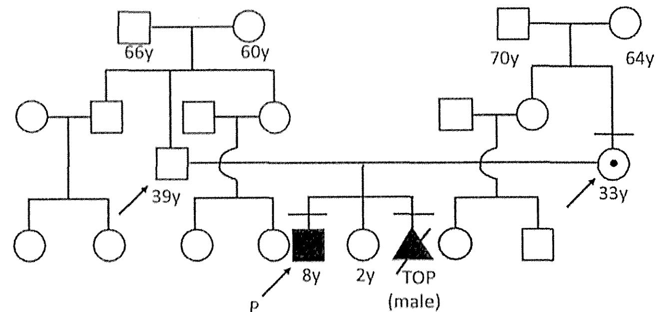

Fig. 2 A pedigree of the patient carrying the L1CAM mutation. A chromosome with the C924T transition was detected in the patient and her two sons. An arrow with P indicates the proband. Only arrow indicates the consultands who are seeking genetic counseling. TOP, termination of pregnancy.

More than 200 *L1CAM* mutations/polymorphisms have been found in unrelated XLH families around the world, of which 51 have been reported in 56 unrelated XLH families within Japan (Yamasaki et al. 2011).

XLH occurs in approximately 5% of all congenital hydrocephalus cases, and the prognosis for XLH is extremely poor (Schrander-Stumpel and Fryns 1998). Neuropathological and molecular data from congenital hydrocephalus cases found that as many as 41% (57/138) had deleterious *L1CAM* mutations (Adle-Biassette et al. 2013). Although it is possible to detect fetal ventriculomegaly by an ultrasound examination, it is extremely difficult to diagnose a congenital hydrocephalus by such means before the 18th week of gestation. In Japan, a termination of pregnancy is legally permitted only before the 22nd week of gestation. Kanemura et al. reported that only 21.7% (5/23) of XLH cases were diagnosed by ultrasound examination prior to the 22nd week of gestation (Kanemura et al. 2006). In our current report of an aborted case, a ventriculomegaly could not be detected at the 18th week of gestation, whereas the XLH-associated *L1CAM* mutation could be definitively detected by a DNA analysis conducted at the 17th week of gestation. Genetic analysis before the 22nd weeks of gestation could thus be very effective for assisting parents in determining whether they should continue a pregnancy or not.

In exon 8, 11 mutation sites were reported according to the database of *L1CAM* gene mutations maintained at the University Medical Center of Groningen web site (http://www.l1cammutationdatabase.info/mutationdetails.aspx). However this is the first report that the C924T transition was prenatally detected in male fetus. The C924T transition in exon 8 occurred in the third base of codon 308, which encodes a glycine, but the change creates

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only an amino acid neutral mutation (G308G). Thus, this transition was initially thought to be silent and to have no effect on the protein's sequence. Meanwhile, Du et al. had reported a similar case of XLH with this same "silent" mutation. Du et al. proposed that the mutation could have created a potential 5′ mRNA splice site consensus sequence, which would have resulted in an in-frame deletion of 69 bp from exon 8 and 23 amino acids of the L1CAM protein, although they did not go on to confirm the clinical significance of the C924T mutation (Du et al. 1998). At present, the C924T has been declared as a "disease-causing" site for XLH, according to the above *L1CAM* gene mutation database.

We report on the usefulness of the detection of a familial C924T mutation in the *L1CAM* gene for the prenatal diagnosis of XLH to aid in genetic counseling. In confirmation of this diagnosis, bilateral adducted thumbs were shown after termination at the 18th week of gestation, indicating the likelihood that the fetus had XLH. To the best of our knowledge, this is the first report of a prenatal diagnosis for XLH that was associated with the C924T "silent" mutation. This adds further confirmation that the mutation can be disease causing for XLH.

DISCLOSURE

The authors declare that they have no competing interests.

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NOTE *Immunology*

Up-Regulation of Cluster of Differentiation (CD) 11b Expression on the Surface of Canine Granulocytes with Human Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

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ABSTRACT. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine, sharing a common beta subunit (CDw131) with interleukins 3 and 5. GM-CSF is important for its direct and indirect involvement in host defense. In veterinary medicine, human (h) GM-CSF has been used as a substitute for canine GM-CSF to stimulate canine granulocytes and macrophages. In this study, we compared the effects of three distinct hGM-CSFs produced by bacteria, yeasts and Chinese hamster ovary (CHO) cells with those of *Escherichia* (E) *coli*-produced canine GM-CSF on the cluster of differentiation 11b (CD11b) expression in canine granulocytes. The median effective dose (ED_{50}) of hGM-CSFs from bacteria, yeasts and CHO cells was 3.09, 4.09 and 4.27 *ng*/m*l*, respectively, with no significant difference among three. In contrast, a significant difference was observed between ED_{50} of canine GM-CSF (0.56 *ng*/m*l*) and three hGM-CSFs according to the paired *t*-test ($P<0.05$). We conclude that hGM-CSF can activate canine granulocytes, but the average activity of the three rhGM-CSFs was approximately 15% of that of canine GM-CSF.

KEY WORDS: canine, CD11b, flow cytometry, granulocyte-macrophage colony-stimulating factor, median fluorescence intensity, xenostimulation

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Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is a protein of 144 amino acids (AA), including the signal peptide of 18 AA, and is produced by various types of cells. The protein is monomeric, but its active form basically takes a noncovalent homodimmer in nature. Although GM-CSF is a major cytokine for hemopoiesis like granulocyte colony-stimulating factor, macrophage colony-stimulating factor and erythropoietin, the cytokine has been known to be involved in the enhancement of eosinophil chemotaxis [7], maturation of macrophages and dendritic cells [17], granulocyte activation [1], adjuvant effect [3] and inhibition of apoptosis [4].

Cluster of differentiation molecule 11b (CD11b), known as its integrin α M subunit, consists of macrophage-1 antigen (Mac-1) with CD18. The molecule is expressed in many types of cells, and the CD11b expression on the surfaces of granulocytes and macrophages is increased by their activation, playing an important role in host defense. Mac-1 has been reported to support neutrophil immobilization and migration [6] and is also known as complement receptor 3 (CR3) that binds to iC3b, eliminating pathogens and immune complexes by neutrophils, macrophages and the reticuloendothelial system. CD11b is rapidly elevated by the activation of neutrophils and macrophages, and the amount of CD11b in neutrophils correlates with their activation and inflammation [11].

Clinical trials of the adjuvant therapy and the prevention form leukocytopenia with GM-CSF in veterinary cancer medicine have been started, but the preparation of canine GM-CSF for clinical use is still unavailable. Thus, we just have to choose that of human GM-CSF (hGM-CSF) at the present time. Because hGM-CSF is active in canine cells, it has been empirically employed as a substitute for canine GM-CSF [18, 22]; however, its quantitative activity in canine cells has not been elucidated. Here, we compared the effects of hGM-CSF to those of canine GM-CSF in canine granulocytes and also measured the median effective doses (ED_{50}) of three different rhGM-CSFs in canine granulocytes.

Anti-CD11b (M1/70) conjugated with allophycocyanin-Cy7, Gr-1 with allophycocyanin and anti-human CD14 with phycoerythrin were purchased from BioLegend Co., Ltd. (San Diego, CA, U.S.A.; provided by Tomy Digital, Tokyo, Japan). Molgramostim; *Escherichia (E.) coli*-produced recombinant human GM-CSF (rhGM-CSF), sargramostim produced by yeasts and canine recombinant GM-CSF were obtained from Amoytop Biotech (Xiamen, Fujian, People's Republic of China), Genzyme corporation (Cambridge, MA, U.S.A.) and R&D systems (Minneapolis, MN, U.S.A.), respectively. JCR Pharmaceuticals Co., Ltd. (Akashi, Japan) donated rhGM-CSF produced by Chinese hamster ovary (CHO) cells.

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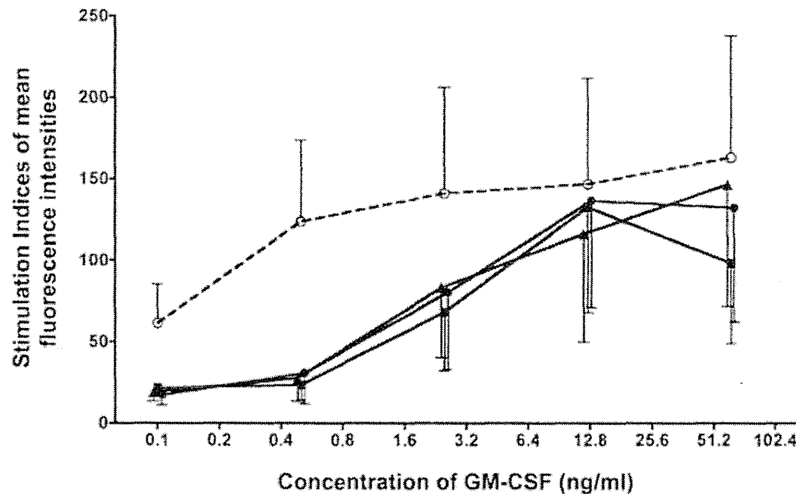

Fig. 1. Dose-response curves of CD11b expression with various granulocyte-macrophage colony-stimulating factors in canine granulocytes. Sigmoid curves represent dose-responses of molgramostim (solid circle), sargramostim (solid square), CHO-produced hGM-CSF (solid triangle) and canine GM-CSF (open circle and broken line). The points and bars show the average values and standard deviations from 4 animals, respectively. X and Y axes show the concentrations of GM-CSF (*ng*/m*l*) and stimulation indices, respectively.

Heparinized canine blood was obtained from 2 male and 2 female beagles for practical trainings of students at Nippon Veterinary and Life Science University. These beagles were individually housed, fed dog chows once a day and drank water *ad libitum*. These bloods were transported to our laboratory and processed at room temperature within 1 hr. Briefly, 100-μl aliquots of the blood were aseptically placed in 2.0-m*l* sterile microtubes, to which various amounts of canine or hGM-CSF were added at final concentrations of 0.02–62.5 *ng*/m*l* or macrophage-serum free medium (macrophage-SFM; Invitrogen Corporation, Carlsbad, CA, U.S.A.) alone. Subsequently, all the samples were incubated for 15 min at a 37°C in a 5% CO_2 incubator without shaking.

After stimulation, antibody cocktail was added to each tube, which was then incubated for 30 min at 4°C. The blood was hemolyzed with 0.15 M ammonium chloride containing 1 mM $KHCO_3$ and 0.1 mM EDTA 4Na (pH 7.3), washed twice with flow cytometer buffer (PBS containing 2% BSA and 0.1% sodium azide) and then fixed in FluoroFix^{TM} buffer (BioLegend), as per the manufacturer's instructions. The cells were re-suspended in 100 μl flow cytometer buffer (PBS containing 2% BSA and 0.05% sodium azide). Data were acquired using FACSArray (BD Bioscience, San Jose, CA, U.S.A.), gating the granulocyte area on a forward vs. side scatter. The median fluorescence intensities (MFIs) of $CD11b^+$ population were obtained under the gate of granulocytes at SSC vs. FSC scatter and $CD14^-$. The indices of MFIs were determined by dividing MFIs from GM-stimulated cultures by MFI from PBS-cultured granulocytes. ED_{50}, determined from MFI values using the probit method, was statistically analyzed using paired *t*-tests at every GM-CSF dose.

Three hGM-CSFs revealed increased CD11b expression on canine granulocytes in a dose-dependent manner (Fig. 1). ED_{50} of molgramostim, sargramostim and hGM-CSF from CHO cells was 3.09, 4.09 and 4.27 *ng*/m*l*, respectively; moreover, no significant difference was observed among these rhGM-CSFs (Table 1). In contrast, ED_{50} of canine rGM-CSF was 0.56 *ng*/m*l*, which was significantly different from the three rhGM-CSFs according to the paired *t*-test results ($P<0.05$). Further, ED_{50} of molgramostim, sargramostim and rhGM-CSF from the CHO cells was 18.1%, 13.7% and 13.1%, respectively, compared with the canine rGM-CSF for canine granulocytes.

GM-CSF is not only an important hemopoietic cytokine, but also involved in the upregulation of the immune system and host-defense [5, 19, 21], because immune cells express its receptor [10, 14]. In experiments using dogs, rhGM-CSF has been employed as a substitute for the canine reagent [2, 16].

GM-CSF activity is usually measured by the proliferation of cells that are GM-CSF-dependent; e.g. TF-1 for hGM-CSF [9]. The detection of augmented CD11b with GM-CSF is rapid and easy. CD11b expression on the surface of neutrophils has been reported to elevate by GM-CSF stimulation [12, 15]. Uchida *et al.* have reported that the quick elevation of CD11b expression on human neutrophils by GM-CSF stimulation was caused by its endogenous molecules but not de novo synthesis [20]. According to a modified Uchida method [20], we detected the activities of rhGM-CSFs in canine neutrophils in a dose-dependent manner. We conclude it may not be a problem to employ rhGM-CSF to canine experiment. This technique doesn't require any GM-CSF-dependent cell line and is applicable to every animal species.

Table 1. Median effective doses of various granulocyte-macrophage colony-stimulating factors in expression of CD11b in canine granulocytes

GM-CSF	ED_{50} (*ng*/m*l*)	Specific activities (units/μg)	Relative activities (%) to canine GM-CSF in ED_{50}
Molgramostim	3.09 ± 1.18 [a]	323.6	18.1
Sargramostim	4.09 ± 1.56 [a]	244.5	13.7
CHO hGM-CSF	4.27 ± 1.51 [a]	234.2	13.1
Canine GM-CSF	0.56 ± 0.46 [a]	1,785.7	–

a) Median effective dose (ED_{50}) of canine granulocyte-macrophage colony-stimulating factor (GM-CSF) significantly differed from those of hGM-CSFs according to the paired *t*-test results (P<0.05).

Furthermore, it has been reported that some mouse cells are not stimulated by hGM-CSF. However, McClure *et al.* proved that rhGM-CSF activated the BaF-B03 mouse cell line transfected with human GM-CSF receptor α subunit gene [13]. The intracytoplasmic region of the subunit did not participate in the signal transduction [23], which suggests that the α subunit plays an important role in binding species-specifically to GM-CSF. Therefore, the α subunit of canine GM-CSF may have an effective affinity to rhGM-CSF, although rhGM-CSFs had a weaker impact on canine granulocytes compared with canine rGM-CSF in this study. Therefore, to obtain an effect equivalent to an expected activity in dogs with hGM-CSF, we must employ an approximately septuplet dose of rhGM-CSF (Table 1). Nevertheless, this indicates that rhGM-CSF can be a valuable tool for a canine study.

In addition, we also compared GM-CSFs from three different sources: *E. coli*, yeasts and CHO cells; although no significant difference was determined in ED_{50} for the three sources, *E. coli*-produced rhGM-CSF (molgramostim) revealed the highest activity. Moreover, Kelleher *et al.* determined that *E. coli*-produced hGM-CSF had higher efficacy with regard to the proliferation of TF-1 cells compared with that of CHO protein [8]. Although we are not able to explain why molgramostim exhibited the highest activity in our study, Kelleher *et al.* suggested that the difference was the result of the higher affinity of *E. coli* protein [8]. Molgramostim is not much different from the other two types investigated without their glycosylation, which may be involved in their 3-D conformation and homodimmer formation and/or interfere with their interactions with GM-CSFR, affecting GM-CSF activity. Thus, the differences in glycosylation may be responsible for their varied activities.

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