Background

Pulmonary alveolar proteinosis (PAP), a rare disorder predominantly affecting the lungs, is characterized by accumulation of surfactant lipids and proteins in the alveoli and terminal bronchioles [1]. PAP is clinically classified into three distinct forms, namely, autoimmune, secondary, and congenital PAP [2]. Autoimmune PAP is associated with disruption of granulocyte/macrophage colony-stimulating factor (GM-CSF) signaling caused by high levels of GM-CSF autoantibody in the lungs [3]. Secondary PAP (sPAP) results from underlying diseases that presumably impair surfactant clearance because of abnormal numbers and functions of alveolar macrophages (AMs). Of the 40 cases of sPAP previously reported by our group [4], 88% (n = 35) involved hematological disorders as underlying diseases. The probability of survival at two years was 46% in cases with sPAP complicating hematological disorders. The median survival time for all cases including 17 patients who died within two years of the sPAP diagnosis was 16 months. Although myelodysplastic syndrome (MDS) is the most frequent underlying disease of sPAP (n = 26, 65%), little information is available on the prognostic impact of development of sPAP on patient outcome.

MDS, a group of clonal hematological stem-cell disorders with ineffective myeloid hematopoiesis and varying degrees of bone marrow failure, is associated with a significant risk of progression to acute myeloid leukemia (AML). Clinical manifestations are variable, from indolent conditions with near-normal life expectancy to forms that rapidly develop into AML [5]. Clarifying much of this heterogeneity, the World Health Organization (WHO) developed a classifications of MDS based on the presence of unilineage or multilineage dysplasia, bone marrow blast cell count, and cytogenetic features: refractory anemia (RA), RA with ringed sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), RCMD with ringed sideroblasts (RCMD-RS), RA with excess of blasts-1 (RAEB-1), and RA with excess of blasts-2 (RAEB-2) [6]. In 2007, Malcovati *et al.* developed a new prognostic scoring system (WHO classification-based prognostic scoring system (WPSS)) according to WHO subgroup, karyotype, and transfusion requirement. Through this system, cases with MDS are classified into very low-, low-, intermediate-, high-, or very high-risk groups [7]. WPSS is a dynamic system that accurately predicts the survival and risk of leukemic evolution in MDS patients at any time during the course of their disease. This time-dependent system seems particularly useful for lower-risk patients and for implementing risk-adapted treatment strategies.

Given the validation of WPSS criteria as a prognostic indicator for the course of MDS, it is applicable in prognosis evaluation of MDS complicated by PAP (MDS/sPAP). In the present study, we evaluated this issue for the first time and found that development of sPAP worsens the prognosis of patients with otherwise low-risk MDS.

Methods

Subjects

Thirty-one patients in Japan who were diagnosed with sPAP with underlying MDS from July 1999 to June 2013 were evaluated. We obtained agreement from all treating physicians for each identified case, according to the Guidelines for Epidemiological Studies by The Ministry of Health, Labour, and Welfare. This was a retrospective cohort study approved by the Ethical Board of Kyorin University (H23-085-01). Cases, part of which were reported previously, were identified retrospectively [8-18].

Diagnosis

Diagnosis of MDS was made according to the 2002 WHO criteria [6]. One patient with unclassified MDS, two patients with myelofibrosis, and two patients with 20% marrow blasts who were considered as having AML were excluded from the study. MDS with isolated chromosome 5 deletion (del(5q)) and marrow blasts of <5% were included. Next, the patients were classified into RA, RARS, RCMD, RAEB-1, and RAEB-2 groups. Karyotypes were classified by using the International System for Cytogenetic Nomenclature Criteria [19]. Diagnosis of PAP was based on the following criteria: 1. histopathological findings from specimens obtained by surgical biopsy or transbronchial lung biopsy, or milk-like appearance with typical cytological findings from bronchoalveolar lavage fluid (BALF); 2. typical high-resolution computed tomography (HRCT) findings for PAP, such as ground glass opacity, consolidation, and interlobular septal thickening; and 3. negative result for serum GM-CSF autoantibody.

Classification by WPSS

The WPSS score was calculated according to the method of Malcovati *et al.* [7] The score was calculated from the data of WHO subgroups (RA/RARS/5q−, RCMD/RCMD-RS, RAEB-1, and RAEB-2), karyotype abnormalities categorized according to the International Prognostic Scoring System [20], and transfusion requirement. The same weight (score of ≥1) was assigned to each variable for WHO subgroup, karyotype, and transfusion requirement. Based on the score, patients were classified according to the following five risk groups: very low (score = 0), low (score = 1), intermediate (score = 2), high (score = 3 to 4), and very high (score = 5 to 6). Transfusion dependency was defined as having at least one

red blood cell (RBC) transfusion every eight weeks over a period of four months.

Statistical analysis

The cumulative probability of survival and risk of progression to leukemia were estimated by using the Kaplan–Meier method. Patients undergoing transplantation treatment were censored at the time of the procedure in order to exclude any potential source of bias due to differential treatment. Variable data were analyzed through Kaplan–Meier methods to estimate the cumulative probabilities of overall survival. The difference in the cumulative probabilities within subcategories of patients was compared by using two-sided log rank test. Survival analyze was performed using Cox proportional model with time-dependent covariates to assess the effect of the variables of interest on overall survival.

Numeric data were evaluated for normal distribution and for equal variance by using the Kolmogorov–Smirnov test and Levene's median test, respectively. Nonparametric data are presented as medians. Categorical data are presented as a percentage of the total or numerically, as appropriate. Statistical comparisons of nonparametric data were compared through the Wilcoxon test. Comparisons of categorical data were made with chi-square or Fischer's exact tests. All tests were two-sided. Statistical significance was indicated by p values of <0.05. Data were analyzed by using SPSS 17.0 software for Windows.

Results

From September 1999 to May 2013, we centrally analyzed for GM-CSF autoantibody in the sera of 619 Japanese cases that had been diagnosed as PAP. Of those cases, 561 were positive for the antibody and 58 were negative. In the cases negative for GM-CSF antibody, 51 demonstrated obvious underlying diseases such as hematological disorders, autoimmune diseases, and infectious diseases. As shown in Figure 1, hematological disorders were the most common underlying disease, 31 cases of which involved MDS. These cases were investigated retrospectively in this study.

Demographic data are shown in Table 1. Diagnosis of MDS was performed in accordance with WHO criteria; 19, 1, 5, 3, and 3 cases were RA, RARS, RCMD, RAEB-1, and RAEB-2, respectively. Karyotyping revealed 2 cases of "high-riskes of g e, 24 cases of "intermediate-riskmediatef, and 5 cases of "low-risks disease. As previously reported [10], there was over-representation of trisomy 8, as it was present in 16 cases (51.6%). RBC transfusion dependency was observed in 11 cases.

PAP was diagnosed on the basis of the BALF and HRCT results in 23 cases and by surgical biopsy and HRCT in eight cases. In 23 cases, diagnosis of MDS was done before diagnosis of PAP, whereas eight cases were diagnosed simultaneously.

According to the WPSS, the cases were classified into 2, 11, 7, 9, and 2 cases of very low-, low-, intermediate-, high-, and very high-risk groups, respectively. For statistical analysis, very low-/low-risk groups and intermediate-/high-/very high-risk groups were categorized as "mild MDS" and "severe MDS" DSere iz, respectively (Table 1). There was no difference in sex and age at the diagnosis of MDS and in hemoglobin concentration, absolute neutrophil count, and platelet count between mild and severe MDS cases. Then, the median age at diagnosis of

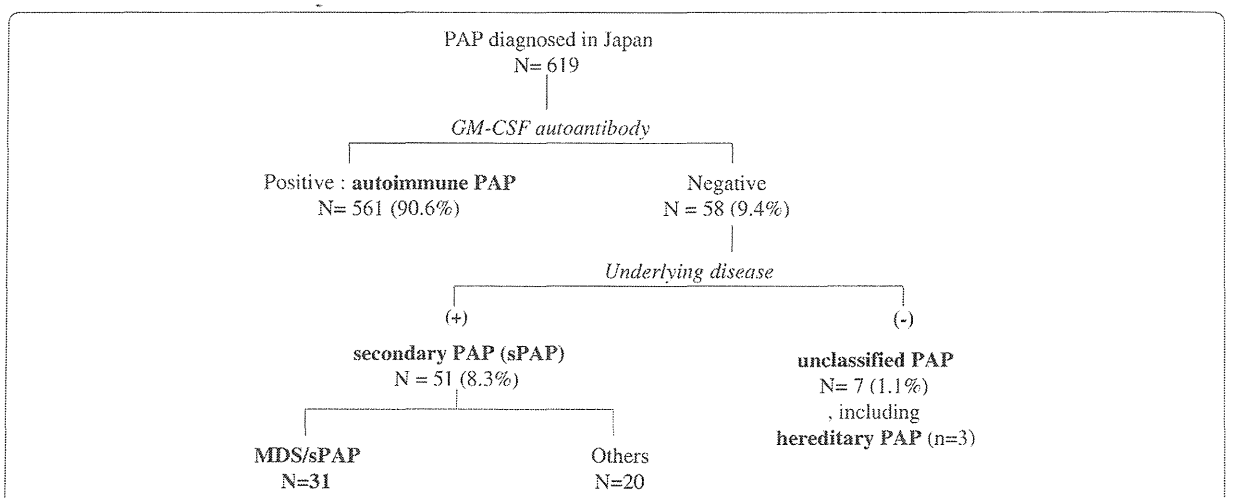

Figure 1 Disposition of patients with pulmonary alveolar proteinosis (PAP) who were enrolled into this study. Participants were stratified according to the presence or absence of granulocyte/macrophage-colony stimulating factor (GM-CSF) autoantibody, and then according to the presence or absence of an underlying disease known to cause PAP.

Table 1 Demographic data at diagnosis of MDS in each group classified according to the WPSS

Median (min.–max.)	Total	<WPSS risk groups> <Very low + low > Mild MDS	<Inter – +high + very high> Severe MDS	*p* value
	(n = 31)	(n = 13)	(n = 18)	
Sex (M/F)	19/12	7/6	12/6	0.71
Age at Dx of MDS	50 (27–75)	45 (30–67)	50 (27–75)	0.54
HbG (g/dl)	9.4 (4.8–16.4)	11.4 (5.5–16.4)	9.0 (4.8–13.9)	0.06
ANC ($\times 10^9$/L)	1.84 (0.01–7.54)	1.46 (0.45–6.97)	2.74 (0.01–7.54)	0.68
PLT ($\times 10^9$/L)	65 (6–219)	45 (14–219)	69 (6–196)	0.31
WHO subgroup: n (%)				
RA/RARS	20 (65)	13 (100)	7 (39)	<0.001
RCMD	5 (16)	0 (0)	5 (28)	0.058
RAEB-1,2	6 (19)	0 (0)	6 (33)	0.02
Karyotype*: n (%)				
Good type	2 (7)	2 (15)	0	0.16
Intermediate type	24 (77)	11 (85)	13 (72)	0.66
Poor type	5 (16)	0 (0)	5 (28)	0.058
RBC transfusion dependency**: n (%)	11 (35)	0 (0)	11 (61)	<0.001

(*) Cytogenetics was as follows. Good type: normal, −Y, del(5q), del(20q); poor type: complex (≥ three abnormalities), chromosome 7 anomalies; and intermediate type: other abnormalities.
(**) RBC transfusion dependency was defined as having at least one RBC transfusion every eight weeks over a period of four months. ANC, absolute neutrophil count; Dx, diagnosis; HbG, hemoglobin; MDS, myelodysplastic syndrome; PLT, platelets; RA, refractory anemia; RAEB, refractory anemia with blasts; RARS, refractory anemia with ringed sideroblasts; RBC, red blood cell; RCMD, refractory anemia with multilineage dysplasia; WHO, World Health Organization; WPSS, WHO classification-based prognostic scoring system.

Table 2 Clinical status at death (n = 17)

	No.	WPSS at diagnosis	WHO criteria at diagnosis of MDS	AML progression 6 (35.3%)	Progression of PAP 11 (64.7%)	Pneumonia 11 (64.7%)
Mild MDS	1	Very low + low	RA			●
	2		RA		●	
	3		RA	●		●
	4		RA		●	●
	5		RA	●		
	6		RA		●	
	7		RA		●	
Severe MDS	8	Intermediate	RA			●
	9		RA		●	●
	10		RA		●	●
	11		RA	●	●	●
	12		RA	●		●
	13		RCMD		●	
	14	High + very high	RA		●	●
	15		RCMD	●	●	
	16		RAEB-1		●	●
	17		RAEB-2	●		●

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; PAP, pulmonary alveolar proteinosis; RA, refractory anemia; RAEB, refractory anemia with blasts; RCMD, refractory anemia with multilineage dysplasia; WHO, World Health Organization; WPSS, WHO classification-based prognostic scoring system.

MDS/sPAP was 51 years, and 84% of cases were symptomatic, with the most common symptoms being fever (45%), dyspnea on effort (42%), and cough (42%). The value of serum Krebs von den Lungen-6 (KL-6) and surfactant protein-D (SP-D) were elevated, and the diffusing capacities of the lung for carbon monoxide (% DLco) were very low in the absence of ventilation disorder in pulmonary function tests. There was no difference in the frequency of respiratory symptoms between patients with mild MDS and those with severe MDS. Serum biomarkers and pulmonary function tests showed no significant difference between the two groups.

Follow-up periods after diagnosis of MDS ranged from five to 254 months (median, 40 months) in all patients (Additional file 1: Figure S1 and Additional file 2: Figure S2). During the follow-up period, 7 patients with mild MDS and 10 patients with severe MDS died, and 2 patients with mild MDS and 4 patients with severe MDS progressed to AML (Table 2). Two and five patients in the mild MDS and severe MDS groups, respectively, underwent transplantation therapies. They were censored at the time of the procedure. The duration from diagnosis of MDS to diagnosis of sPAP was variable, ranging from 0 to 168 months in the mild MDS group and from 0 to 228 months in the severe MDS group (Figure 2A). The median duration of MDS prior to diagnosis of sPAP in the mild MDS group was significantly longer than that in the severe MDS group

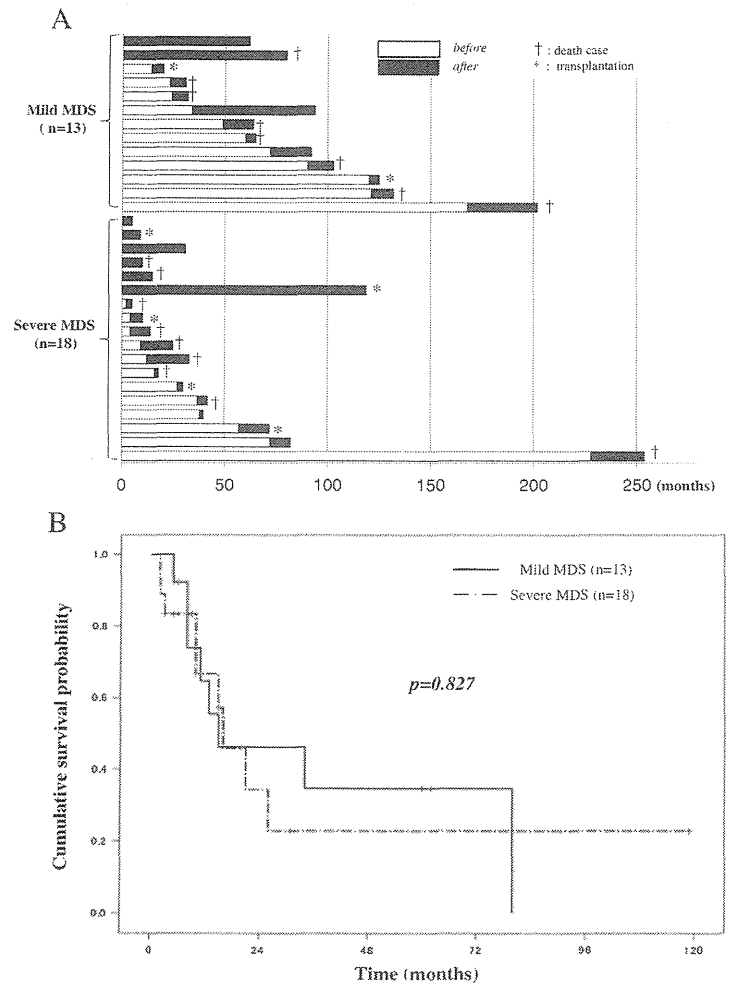

Figure 2 Disease duration of secondary PAP in patients with MDS. A: Duration of disease before or after the diagnosis of secondary pulmonary alveolar proteinosis (sPAP). The horizontal axis indicates the month after the diagnosis of myelodysplastic syndrome. The open column and closed column indicate the duration of disease respectively before and after the diagnosis of sPAP. The case that resulted in death is marked by † and the case that underwent transplantation therapy is marked by *. **B**: Cumulative survival probability after diagnosis of secondary PAP (sPAP) in patient groups with mild myelodysplastic syndrome (MDS) (n = 13) and severe MDS (n = 18). The horizontal axis indicates the month after the diagnosis of sPAP, and the vertical axis indicates the cumulative survival probability.

(p = 0.034). Three patients in the mild MDS group and one in the severe MDS group survived for more than five years after diagnosis of sPAP. Of those, spontaneous remission of sPAP occurred in three cases.

A previous report [7] demonstrated that prediction of survival was dependent on the severity of MDS (as defined by WPSS) at any time of the disease. In contrast, patients with mild MDS in our study had cumulative survival probability similar to that of patients with severe MDS (Figure 2B, p = 0.827). This similarity may be due to poor prognosis of mild MDS after diagnosis of PAP. The cumulative survival probability curves of mild and severe MDS groups with median survivals of 13 and 15 months, respectively, are comparable. Concerning causes for the death of seven patients in the mild MDS group were progression to AML in two cases, PAP exacerbation in four cases, and fatal infectious disease in three cases (Table 2). Concerning causes for the death of 10 patients in the severe MDS group were progression to AML in 4 cases, PAP exacerbation in 7 cases, and fatal infectious disease in 8 cases. Fatal infectious diseases consistently arose from severe pneumonia with (n = 4) or without systemic sepsis, suggesting that progression of PAP was the major cause of death in both mild and severe MDS patients. These results suggest that occurrence of sPAP principally reduced the survival of patients with mild MDS. Pathogens isolated in the fatal cases were identified as *Aspergillus* species (four cases), *Pseudomonas aeruginosa* (four cases), and non-tuberculosis *Mycobacterium* species (four cases).

By performing univariate analysis using Cox proportional model we then searched for potential prognostic factors. Age, sex, respiratory symptoms, history of respiratory failure, diagnostic procedure for sPAP, and MDS group (mild or severe), were not associated with survival at the time of diagnosis of sPAP (Table 3). Treatment with

Table 3 Univariate analysis of overall survival after diagnosis of sPAP in MDS

Variable at diagnosis of sPAP	(n)	75% of OS (months)	50% of OS (months)	HR (95% CI)	*P* value
Age: 51 yrs or younger	16	8	16		
Older than 52 yrs	15	10	15	1.29 (0.48-3.41)	0.607
Gender: Male	19	10	16		
Female	12	11	21	1.12 (0.43-2.94)	0.804
MDS group: mild	13	10	15		
severe	18	11	16	1.11 (0.42-2.95)	0.830
Symptoms: (−)	5	26	26		
(+)	26	6	15	1.50 (0.33-6.65)	0.592
Dx procedure: Bronchoscopy	23	11	13		
Surgical biopsy	8	15	26	0.69 (0.23-2.04)	0.507
Respiratory failure: (−)	21	11	26		
(+)	10	5	10	2.18 (0.77-6.22)	0.142
Use of corticosteroid: (−)	16	16	80		
(+)	15	10	13	3.20 (1.09-9.38)	0.034
Serum KL-6 (U/ml): < 1960	16	13	26		
1960 ≦	15	5	15	1.52 (0.58-4.00)	0.389
Serum SP-D (ng/ml): < 147	15	10	26		
147 ≦	15	8	15	1.80 (0.62-5.22)	0.278
Serum SP-A (ng/ml): < 79	16	11	26		
79 ≦	14	5	15	2.79 (0.965-8.06)	0.058
%VC: 87 ≦	11	15	34		
< 87	11	8	15	3.27 (0.79-13.52)	0.101
FEV1%: 86 ≦	12	8	16		
< 86	10	13	34	0.52 (0.14-1.87)	0.322
%DLco: 44 ≦	9	21	34		
< 44	8	5	13	9.98 (1.03-96.11)	0.046

CI indicates confidence interval; OS, overall survival; DLco, diffusing capacity of the lung for carbon monoxide; Dx, diagnosis; FEV, forced expiratory volume; HR, hazard ratio; KL-6, krebs von den lungen-6; MDS, myelodysplastic syndrome; SP-A, surfactant protein -A; sPAP, secondary pulmonary alveolar proteinosis; SP-D, surfactant protein -D; VC, vital capacity.

corticosteroids was associated with poor survival (p = 0.024) (Figure 3A). However, the number of patients treated with steroid therapy did not differ between mild and severe MDS groups. A %DLco of <44% (Figure 3B) predicted poor prognosis (p = 0.019), whereas %vital capacity (%VC), forced expiratory volume (FEV) 1.0%, serum KL-6, SP-D, and surfactant protein-A (SP-A) did not.

Discussion

For the first time, we evaluated the prognosis of MDS/sPAP in a substantial cohort of patients, comparing mild and severe MDS as classified according to WPSS criteria. Our data demonstrate that the duration from diagnosis of MDS to diagnosis of sPAP was longer in mild MDS than that in severe MDS, but the survival probability was similar after the diagnosis of sPAP regardless of MDS severity. As a whole, occurrence of PAP appeared to worsen the prognosis of patients with mild MDS. This result is supported by the fact that the major cause of death was not MDS-associated but rather sPAP-associated respiratory failure or infections. Prior to our report, 21 cases with MDS/sPAP have been reported

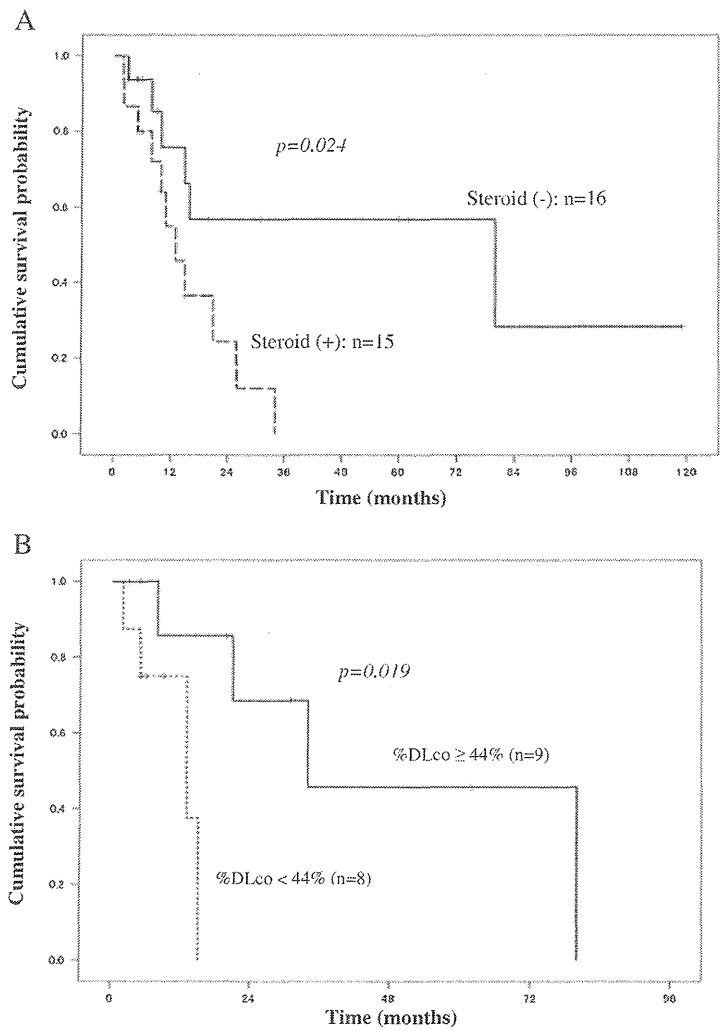

Figure 3 Risk factors for the prognosis of secondary PAP in patients with MDS. A: Cumulative survival probability after diagnosis of secondary PAP (sPAP) in myelodysplastic syndrome (MDS) cases with steroid therapy (n = 15) and in MDS cases without steroid therapy (n = 16). The horizontal axis indicates the month after the diagnosis of sPAP, and the vertical axis indicates the cumulative survival probability. The number of cases that received steroid therapy in the mild and severe MDS groups were six (46%) and nine (59%), respectively. **B**: Cumulative survival probability after diagnosis of secondary PAP (sPAP) in myelodysplastic syndrome (MDS) cases with diffusing capacity of the lung for carbon monoxide (%DLco) of <44% (n = 8) and in MDS cases with%DLco of >44% (n = 9). The horizontal axis indicates month after the diagnosis of sPAP, and the vertical axis indicates the cumulative survival probability.

[8-18]. Most reports [9-18,21-24] describe a single case, whereas only two publications report multiple cases [8,25]. The clinical course described in these reports suggest a poor prognosis for MDS/sPAP patients, but no prior report clearly quantifies the outlook for these patients and compares this to a predicted outcome by using validated prognostic scores such as WPSS. MDS/sPAP is so rare a disease that neither pulmonologists nor hematologists encounter such patients very often. In fact, in more than 10 years, 2 to 5 cases were diagnosed as MDS/sPAP annually in our analyses for serum GM-CSF autoantibody in over 600 diagnosed PAP cases from all over Japan; thus, we have finally reached cumulative 31 cases with MDS/sPAP.

According to the WHO criteria, 20 of the 31 cases were RA/RARS, 5 cases were RCMD, and 6 cases were RAEB1-2. The proportion of the number for each subtype in the total number of cases was comparable to literature data in terms of frequency and subtype distribution, suggesting that the risk of PAP complicating MDS is similar regardless of the subtype of MDS. It is speculated that AMs in patients with MDS derive from abnormal bone marrow precursor cells and are defective in both surfactant homeostasis and host defense, hence the progression of PAP and PAP-associated infections even in cases with mild MDS. Previous studies reported that in the absence of complicating sPAP, the five-year survival probability for patients with RA and RARS was 74% [26], whereas our cases with RA plus RARS had substantially inferior prognosis (0.69) (Additional file 2: Figure S2).

It is noteworthy that treatment with corticosteroids was associated with a markedly inferior prognosis. In Japan, steroid therapy often has been used for PAP but no evidence of its efficacy has been found. To our surprise, 15 of 31 cases had undergone steroid therapy during the course of sPAP. Our data reveal that steroid-treated patients had worse prognosis than did patients without steroid therapy. Given that the predominant cause of death was infective complications potentially exacerbated by steroid-related immunosuppression, these data clearly caution against the use of steroid therapy in such patients.

Treatment of MDS/sPAP should be directed toward the underlying malignancy, i.e., MDS. It should also aim at restoring hematopoietic function, either through allogeneic bone marrow transplant, which has curative potential for both MDS and sPAP, or through hypomethylating agent therapy for MDS, which can restore numerical hematologic parameters. However, functional cellular defects will likely remain, as such therapy does not necessarily eradicate the underlying clone, but rather enhances cellular differentiation [27,28]. Nevertheless, seven cases with MDS/sPAP to date in our cohort had undergone transplantation therapy. Of those, three patients died of pneumonia within three months of the transplantation therapy. Therefore, we do not have any convincing evidence to recommend transplantation therapy in the early stages of the disease. Although whole-lung lavage and segmental bronchial lavage were performed in 10 patients, only 3 cases showed the efficacy of lung-lavage therapy.

Infection often coexists with MDS/sPAP, although the causal relationship between PAP and infection is not clear. Superimposed infection accounts for a significant degree of morbidity and mortality in patients with sPAP. In the present study, 11 among 17 cases with fatal outcomes developed fatal infectious diseases. Considering that this complication was observed in the mild MDS and severe MDS groups, pneumonia accompanied with sPAP might be the trigger of fatal infection. Nevertheless, the present number of cases (31) is too small for accurate prognosis evaluation of MDS/sPAP; future international collaboration may be necessary to overcome this difficulty.

Conclusions

Complication of sPAP is an important risk factor in the prognosis of MDS. We believe that the present data will contribute to the management and treatment of the disease.

Additional files

Additional file 1: Figure S1. Survival curves after diagnosis of MDS in each mild and severe MDS.

Additional file 2: Figure S2. Survival curves in each MDS groups classified by WHO-criteria.

Abbreviations

AM: Alveolar macrophage; AML: Acute myeloid leukemia; ANC: Absolute neutrophil count; BALF: Bronchoalveolar lavage fluid; CEA: Carcinoembryonic antigen; CT: Computed tomography; DLco: Diffusing capacity of the lung for carbon monoxide; Dx: Diagnosis; FEV: Forced expiratory volume; GM-CSF: Granulocyte macrophage colony-stimulating factor; HbG: Hemoglobin; HRCT: High-resolution computed tomography; KL-6: Krebs von den Lungen-6; MDS: Myelodysplastic syndrome; PAP: Pulmonary alveolar proteinosis; PLT: Platelets; RA: Refractory anemia; RARS: Refractory anemia with ringed sideroblasts; RAEB: Refractory anemia with blasts; RBC: Red blood cell; RCMD: Refractory anemia with multilineage dysplasia; SD: Standard deviation; SP-A: Surfactant protein-A; sPAP: Secondary pulmonary alveolar proteinosis; SP-D: Surfactant protein-D; VC: Vital capacity; WHO: World Health Organization; WPSS: WHO classification-based prognostic scoring system.

Competing interests

Financial/non-financial competing interests

The authors report no potential conflicts of interest with any companies or organizations whose products or services are mentioned in this article.

Authors' contributions

The authors take responsibility and vouch for the completeness and accuracy of the data and analyses. HI and KN are the guarantors of the entire manuscript. HI and JS contributed to the study concept and design, coordination of data acquisition, and writing of the article. RT, YI, NU, AN, YK,

and TS contributed to the data analysis. KT, TT, YI, MH, TI, and HG contributed to the interpretation of the data and writing of the article. KN participated in writing and critically revising the manuscript. All authors read and approved the final manuscript.

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REVIEW ARTICLE
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Novel aspects on the pathogenesis of *Mycoplasma pneumoniae* pneumonia and therapeutic implications

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Mycoplasma pneumoniae (Mp) is a leading cause of community acquired pneumonia. Knowledge regarding Mp pneumonia obtained from animal models or human subjects has been discussed in many different reports. Accumulated expertise concerning this critical issue has been hard to apply clinically, and potential problems may remain undiscovered. Therefore, our multidisciplinary team extensively reviewed the literature regarding Mp pneumonia, and compared findings from animal models with those from human subjects. In human beings, the characteristic pathological features of Mp pneumonia have been reported as alveolar infiltration with neutrophils and lymphocytes and lymphocyte/plasma cell infiltrates in the peri-bronchovascular area. Herein, we demonstrated the novel aspects of Mp pneumonia that the severity of the Mp pneumonia seemed to depend on the host innate immunity to the Mp, which might be accelerated by antecedent Mp exposure (re-exposure or latent respiratory infection) through up-regulation of Toll-like receptor 2 expression on bronchial epithelial cells and alveolar macrophages. The macrolides therapy might be beneficial for the patients with macrolide-resistant Mp pneumonia via not bacteriological but immunomodulative effects. This exhaustive review focuses on pathogenesis and extends to some therapeutic implications such as clarithromycin, and discusses the various diverse aspects of Mp pneumonia. It is our hope that this might lead to new insights into this common respiratory disease.

Keywords: *Mycoplasma pneumoniae* pneumonia, animal models, epidemiology, pathology, pathogenesis

INTRODUCTION

Mycoplasma pneumoniae (Mp) was first isolated in tissue culture from the sputum of a patient with primary atypical pneumonia by Eaton et al. (1944). This "Eaton's agent" was shown to be a *Mycoplasma* species in 1961. Chanock et al. succeeded in culturing Eaton's agent in mammalian cell-free medium and proposed the taxonomic designation Mp in 1963 (Chanock et al., 1962; Chanock, 1963). Mp is a unique organism that lacks a cell wall in any circumstances, and does not need a host cell for replication. This organism causes a variety of clinical presentations, from self-limiting to life-threatening. The disease severity seems to depend on the degree of host's defenses. In this review, we focused on the pathogenesis of Mp pneumonia from the perspective of host defenses, based on findings from our mouse models.

EPIDEMIOLOGY

Mp is one of the most common pathogens of community-acquired pneumonia (CAP) in adults (**Table 1**). In general, both regional differences and varying periods of surveillance may influence the results of etiological studies of infectious diseases. **Table 1** summarizes the proportions of adult Mp pneumonia among CAP populations enrolled in several large-scale studies conducted in various countries (Marston et al., 1997; Ngeow et al., 2005; Arnold et al., 2007; Von Baum et al., 2009; Cilloniz et al., 2011). Mp pneumonia accounted for 10.6–17.0 and 3.0–20.8% of CAP in out- or in-patients settings, respectively, and the frequency of ICU admission was relatively low (2–3.6%). Arnold et al. showed that Mp is the most common atypical pneumonia pathogen, accounting for 11–15% of CAP throughout the world (Arnold et al., 2007). Serological studies in Denmark over a 50-year period showed that Mp infections exhibit epidemic periodicity every 3–5 years, but this trend now seems to be getting obscured (Lind et al., 1997). Mp pneumonia occurs at any age, but the incidence is less common in elderly, as compared with young, adults (Lim et al., 2009), and is highest among school-aged children (Foy et al., 1979).

Macrolides were recommended for treatment of microbiologically defined Mp pneumonia. However, macrolide-resistant

Table 1 | Prevalence of *Mycoplasma pneumoniae* pneumonia in CAP.

Author	Country	Year	*N*	Out-Pts%	Ward%	ICU%	Total%	Mortality%
Cilloniz	Spain	1996–2008	1463	17	3	2	4	3.1
Ngeow	Asia	2001–2002	926	ND	ND	3.6	11.4	ND
Baum	German	2002–2006	4532	10.6	4.7		6.8	0.7
Marston	USA	1991	1938	ND	20.8** (5.4*)		20.8* (5.4*)	ND
Arnold	Whole world	2001–2006	4337	ND	12		12	ND

(Marston et al., 1994; Ngeow, 2004; Arnold et al., 2007; Baum et al., 2009; Cilloniz et al., 2011).

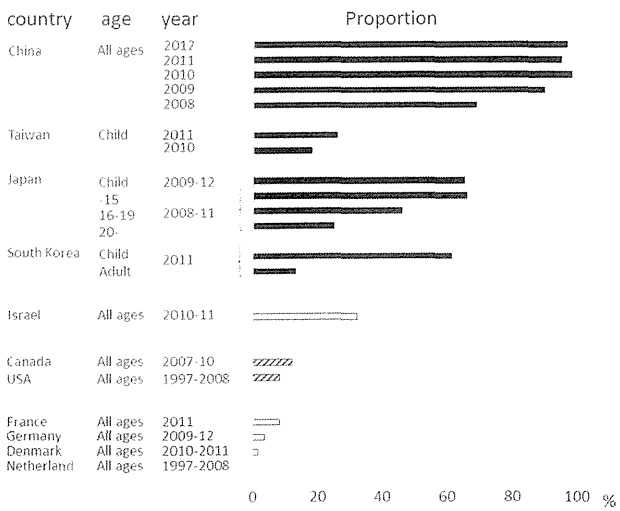
*definite case.

**definitive and possible cases.

N, number; ND, not determined; Out-Pts, out patients.

Mp was isolated from Japanese children, and the incidence was increasing in the early 2000s (Matsuoka et al., 2004). There was a major concern that macrolide-resistant Mp had increased locally and was spreading throughout the world. In East Asia, macrolide-resistant Mp rapidly increased and became the cause of the majority of clinically-proven Mp in both children and adults. The prevalence of macrolide-resistant Mp varies among countries and age groups (Averbuch et al., 2011; Akaike et al., 2012; Miyashita et al., 2012; Spuesens et al., 2012; Ursum et al., 2012; Yamada et al., 2012; Yoo et al., 2012; Dumke et al., 2013; Eshaghi et al., 2013; Pereyre et al., 2013; Wu et al., 2013; Zhao et al., 2013) (**Table 2**). For example, over 90% of isolated Mp in China was macrolide resistant, while no macrolide-resistant Mp was found in the Netherlands. Generally, it became highly prevalent in East Asian countries including China, Japan and South Korea, while being a medium or low prevalent in North America and Europe, respectively. Macrolide-resistant Mp is reportedly more prevalent in children, and the predominant point mutation found was A2063G in domain V of 23S rRNA. Aside from geographical and racial differences between individual studies, the application of different diagnostic techniques or criteria might affect the epidemiology of Mp pneumonia in each study.

Table 2 | Proportions of macrolide-resistant *Mycoplasma pneumonia*.



HUMAN PATHOLOGY AND BRONCHOALVEOLAR LAVAGE FLUID

PATHOLOGY

Studies focused on the pathological description of human Mp pneumonia have rarely been reported. However, pathological examinations have been conducted on several different types of specimens that were sampled using different techniques; e.g., autopsy specimens (Parker et al., 1947; Maisel et al., 1967; Benisch et al., 1972; Meyers and Hirschman, 1972; Halal et al., 1977; Kaufman et al., 1980; Koletsky and Weinstein, 1980), open lung biopsy specimens (Coultas et al., 1986; Rollins et al., 1986; Llibre et al., 1997; Ebnother et al., 2001; Wachowski et al., 2003), video-assisted thoracic surgery (VATS) specimens (Chan et al., 1999) and transbronchial lung biopsy specimens (Ganick et al., 1980; Nakajima et al., 1996; Ohmichi et al., 1998). According to these reports, the most characteristic pathological feature of human Mp pneumonia is a marked plasma cell-rich lymphocytic infiltration in the peri-bronchovascular areas (PBVAs), with accumulations of macrophages, neutrophils, and lymphocytes in the alveolar spaces (Parker et al., 1947; Coultas et al., 1986; Rollins et al., 1986). The presence of plasma cells in PBVAs might reflect up-regulation of humoral immunity via Mp infection.

BRONCHOALVEOLAR LAVAGE FLUID (BALF) FINDINGS

There have been several case series focused on BALF obtained from human Mp pneumonia patients (Hayashi et al., 1986, 1993, 1998; Yano et al., 2001); those studies demonstrated varying levels of monocytes, polymorphonuclear leukocytes (PMNs), lymphocytes, eosinophils, and total cell counts. Among them, PMNs and lymphocytes counts were relatively more increased than the other cell types. The CD4 to CD8 ratios in the BALF were also elevated, and ranged from 2.1 (Hayashi et al., 1986) to 3.5 ± 2.1 (Hayashi et al., 1993), irrespective of the sampling timing.

PATHOGENESIS

ANIMAL MODELS

The incidence of Mp pneumonia is relatively low among the elderly over 70 years old or children less than 5 years old. This led to the hypothesis that elderly persons must be repeatedly exposed to and respond immunologically to the organism with clinical or subclinical progression. Indeed, as for cellular immunity, Brunner et al. have suggested that the occurrence of clinical

disease in adults is favored by prior sensitization induced by infection at an early age, causing large or small mononuclear cell reactions (Brunner et al., 1973). This cellular response, lasting several years, could be proved by Mp antigen-induced lymphocyte transformation of cell suspensions from previously infected patients (Biberfeld et al., 1974; Biberfeld, 1974). It is important for us to understand immune responses attributed to Mp pneumonia.

We designed five different mouse models for Mp pneumonia (**Figure 1**) to examine the resulting pathology in animals having various immune status (Saraya et al., 2007b, 2011; Saraya, 2013). Animals were peritoneally immunized with various regimens (one per model) once a week (on days −14 and −7), then 1 week after the last immunization the animals were intratracheally (IT) challenged with sonicated Mp antigen, as previously reported (Saraya et al., 2011). Among those models, only groups immunized with Mp antigen and alum adjuvant (**Figure 1E**) or CpG (**Figure 1C**) developed severe lymphocytic infiltration into PBVAs at 96 h after IT (**Figures 2C,E**) while, no inflammatory cells were seen on models A and B (**Figures 2A,B**). However, the pathognomonic feature for human Mp pneumonia was reconstructed only in models D and E, in which lymphoplasmacytic infiltration into PBVAs occurred 96 h post-IT (**Figures 2D,E**). Those results suggest that enhanced host immune responses, as occurred in models C and E, against Mp antigen are required for persistent inflammation in the lung, as well as Th2 characteristics (produced by use of Th2 adjuvant, as in models D and E) causing plasma cell infiltration into the PBVAs, but not Th1 characteristics (produced by use of the Th1 adjuvant, CPG, as in the model depicted in **Figure 1C**). Aluminum hydroxide adjuvant, named alum, is well-known for initiating strong antigen-specific Th2 responses in the absence of interleukin(IL)-4- or IL-13-mediated signaling (Brewer et al., 1999); Th2 predominant characteristics might be required to generate typical Mp pneumonia, even in humans. Previous studies showed that the histopathological score of Mp pneumonia is significantly higher in infected BALB/c mice (Th2 predominant) than in C57BL/6 mice (Th1 predominant) through the late phase, suggesting differences in host reactions against intranasally-inoculated live Mp (Fonseca-Aten et al., 2005). Tanaka et al. (1996) describe the different pathological findings in an *M. pulmonis*–infected mouse model for treatment with IL-2 (Th1 up-regulated) vs. cyclosporine A (Th1 down-regulated).

Thus, the severity of Mp pneumonia seems to depend on the host immune response to the infection through a complexity of various mechanisms, including an allergic reaction to Mp, Mp virulence, host defenses, and polarization toward Th1 or Th2 predominance, to name a few. In the context of allergic reaction, IgE antibodies specific to Mp were detected in serum samples from patients with Mp pneumonia, suggestive of IgE-mediated hypersensitivity (Tipirneni et al., 1980; Yano et al., 1994; Seggev et al., 1996) as well as an involvement in asthma attacks (Henderson et al., 1979; Biscardi et al., 2004). In this review, we will further discuss the pathomechanisms of Mp pneumonia from the perspective of the virulence of Mp and presumed host defenses based on findings obtained from our experimental mouse models.

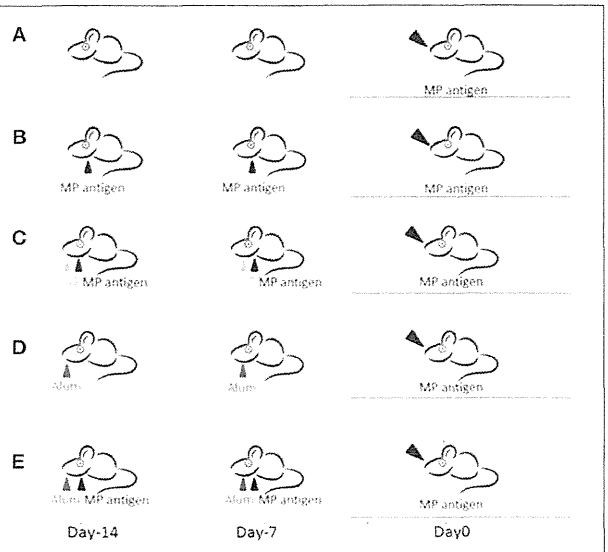


FIGURE 1 | Female BALB/c (7-week-old) mice were inoculated intratracheally with Mp antigen with or without pre-immunization. Pre-immunization was carried out by intraperitoneal injection at 7 and 14 days prior to the intratracheal (IT) challenge. Model A: IT without pre-immunization. Model B: IT after twice pre-immunizing with Mp antigen alone. Model C: IT after twice pre-immunizing with Mp antigen plus CpG. Model D: IT after twice pre-immunizing with alum alone. Model E: IT after twice pre-immunizing with Mp antigen plus alum. One week following the last immunization, mice underwent IT with 50 μg of Mp antigen. Bronchoalveolar lavage fluid (BALF) and lung specimens were obtained before this process and 8, 24, 48, 96, and 168 h after IT. Mp: mycoplasma.

VIRULENCE OF MP

Lipoproteins

Lipoproteins from various Mycoplasma species have potent inflammatory properties. Three lipoproteins/lipopeptides of *M. fermentans* origin, macrophage-activating lipopeptide-2 (MALP-2), P48, and M161Ag (identical to MALP-404), reportedly modulate the host immune system via Toll-like receptor (TLR)-2/TLR-6 signaling (Takeuchi et al., 2000; Luhrmann et al., 2002; Seya and Matsumoto, 2002). Genes for more than 30 different Mp lipoproteins have been reported (Himmelreich et al., 1997). Shimizu et al. reported that the mycoplasma-derived lipoproteins N-ALP1/N-ALP2 (Shimizu et al., 2008) and F_0F_1-ATPase (Shimizu et al., 2005) activated NF-kβ via TLR-1, 2 or TLR-1, 2, 6 signaling, respectively. Stimulation of these TLRs has been known to be related to production of chemokines (Brant and Fabisiak, 2008; Andrews et al., 2013) that promote lymphocyte and neutrophil trafficking and inflammation in the lung.

CARDS (Community Acquired Respiratory Distress Syndrome) toxin

Kannan et al. first demonstrated the possibility that Mp produces the CARDS toxin that is involved in the mediation of disease (Kannan et al., 2005). The CARDS toxin is an ADP-ribosylating and vacuolating toxin, with homology to the S1 subunit of pertussis toxin, that has a high affinity for surfactant protein-A, suggesting a physiological role for the toxin in the pulmonary compartment. In mice, intranasal inoculation

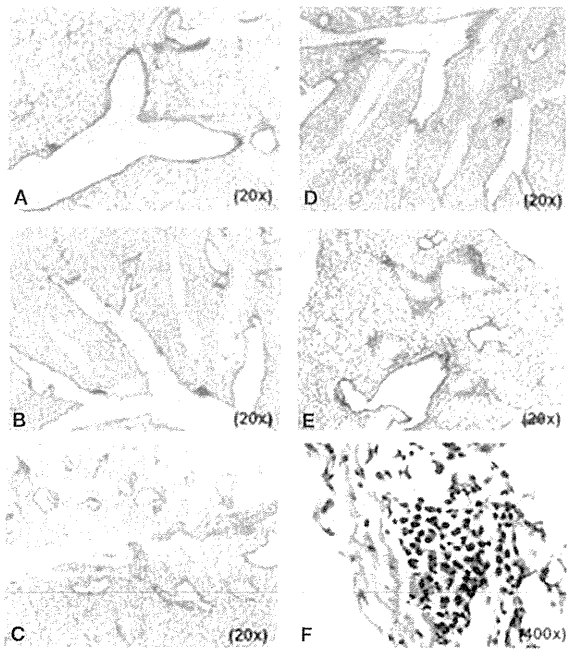


FIGURE 2 | Histopathological examination of lung specimens. At 96 h post-intratracheal (IT) challenge, no inflammatory cells were seen in model **(A)** A or **(B)** B specimens. Mild to moderate lymphocyte infiltration was observed in the peribronchovascular area (PBVA) of **(D)** model D tissue. Models **(C)** C and **(E)** E had more severe lymphocyte infiltration in the PBVA. Plasmacyte infiltration within the PBVA was only recognized in models D and E, and the number of infiltrating plasmacytes was significantly higher in model E than model D. **(F)** High power magnification of E. Hemotoxylin and eosin stain. **A–F**, 200x; **E**, 400x.

of recombinant CARDS toxin caused an increased level of pro-inflammatory cytokines IL-1α, 1β, 6, 12, 17, Tumor necrosis factor(TNF)-α, and Interferon-gamma (IFN) -γ together with elevation of Keratinocyte chemoattractant(KC), IL-8, regulated on activation, normal T cell expressed and secreted (RANTES), and G-CSF (Hardy et al., 2009). However, to our knowledge, there have been no reports of CARDS toxin identified in human respiratory specimens.

Other factors

Mp produces a soluble hemolysin (Somerson et al., 1963, 1965), hydrogen peroxide and superoxide radicals, which produce oxidative stress in the respiratory epithelium, resulting in both structural and functional deterioration of cilia (Waites and Talkington, 2004). Stimulation of human respiratory epithelial cells (A549 cells) *in vitro* with Mp lysate (MPL) induced IL-8 production (Sohn et al., 2005). MPL induced IL-8 release in a time- and dose-dependent manner together with activation of extracellular signal-regulated kinase (ERK), which was inhibited by PD98059, a specific inhibitor of ERK. Chmura et al. (2003) reported that the Mp membrane fraction induced IL-8 on BEAS-2B human bronchial epithelial cells. Our report (Hirao et al., 2011) also demonstrated activation of mitogen-activated protein kinase (MAPKs) on the alveolar macrophage-like cell line, RAW264.6, by stimulation with Mp antigen, as confirmed by significant suppression of IL-6 and TNF-α production after preceding treatment with an MAPKs inhibitor such as parthenolide (PAR: NF-kB inhibitor), SB20580 (SB, p38-linked signal of inhibitor), or LY294002 (LY, PI-3K inhibitor). Thus, Mp antigen or live Mp can induce inflammatory cytokines in bronchial epithelial cells and in alveolar macrophages (AMs).

HOST DEFENSES

Cellular immunity

Biberfeld et al. reported that the peripheral lymphocyte response to a sonicate of Mp organisms or a membrane fraction was significantly higher in recently infected patients than in healthy patients (Biberfeld et al., 1974). The positive responsiveness to sonicated Mp antigen was demonstrable up to 10 years after infection. Others also reported on the *in vitro* response of human peripheral lymphocytes to Mp antigen (Fernald, 1972; Biberfeld, 1974), while tuberculin anergy in patients with Mp pneumonia was noted soon or fairly soon after onset. This has been speculatively explained by the possibility that (1) lymphocytes and macrophages needed for the skin reaction to tuberculin are engaged in the immune response to the infecting agent, or (2) a transient change of the T lymphocyte population occurs (Biberfeld and Sterner, 1976). Tanaka et al. reported that the rate of positive tuberculin tests during the acute stage of Mp pneumonia was higher in patients with the nodular type of pulmonary lesions on thoracic computed tomography than those having the consolidation pattern. This finding suggests that the level of current cell-mediated immunity might influence the pattern of pulmonary lesions. Another study showed that delayed hypersensitivity was noted on skin testing with Mp antigen of patients with Mp pneumonia (Mizutani et al., 1971).

However, to our knowledge, no direct evidence from patients with Mp pneumonia has been reported regarding the reactivity of BALF lymphocytes to Mp antigen. In other words, it is still under debate whether the lung inflammation of Mp pneumonia is a specific reaction to the Mp antigen.

In consideration of this question, Saraya et al. (2011) demonstrated a lack of specific response of lymphocytes in the BALF to Mp antigen 96 h post-IT using the 3H-thymidine uptake test in an Mp pneumonia mouse model (**Figures 1D,E**). The BALF cells in the lymphocyte gate were 35.8% CD3 positive and 57.6% CD3 negative. Among the CD3 positive cells, CD4−/CD8− cells were predominant. The CD4 to CD8 ratio was 0.02, which was a lower value than that of human Mp pneumonia patients (Hayashi et al., 1986, 1993), and the CD8 positive cells consisted of naïve cells ($CD62L+^{hi}$/ $CD44+^{lo}$), effector memory cells ($CD62L+^{lo}/CD44+^{hi}$), and central memory cells ($CD62L+^{hi}/CD44+^{hi}$), in that order (Saraya et al., 2007a) (**Figure 3**). Cellular immunity seemed to play an important role in development of Mp pneumonia (Foy et al., 1973; Broughton, 1986); the results given above might indicate that non-specific reactions to Mp antigen govern the severity of lung inflammation.

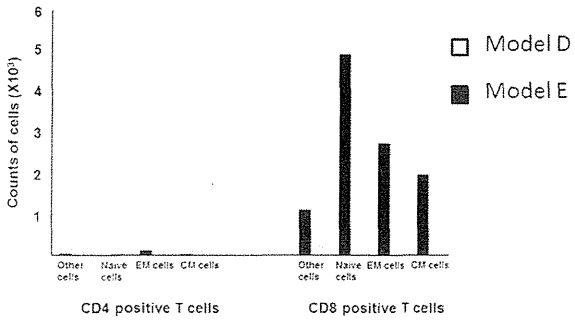


FIGURE 3 | Flow cytometry analyses of CD3 positive lymphocytes from the BALF of models D (□) and E (■) at 96 h post-intratracheal challenge. CD8-positive cells predominated, and consisted of naïve cells (CD62L+^{hi}/ CD44+^{lo}), effector memory cells (CD62L+^{lo}/CD44+^{hi}), and central memory cells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

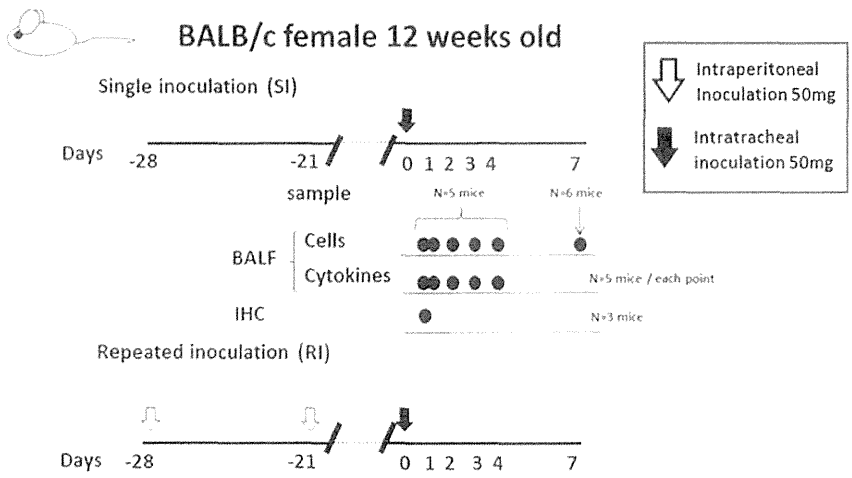


FIGURE 4 | The "no adjuvant used" model was created by intraperitoneally immunizing with only Mp antigen twice, on days −28 and −21 (for the RI, repeated inoculation, group) or without pre-treatment (for the SI, single inoculation, group), followed by intratracheal challenge with Mp antigen at day 0 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

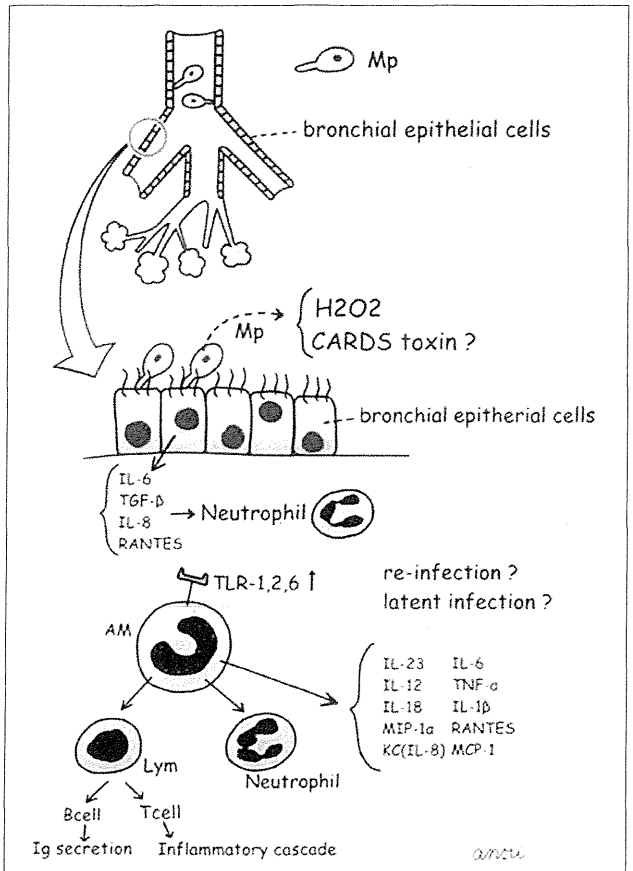


FIGURE 5 | Postulated schema for pathogenesis of human Mp pneumonia. CARDS, Community Acquired Respiratory Distress Syndrome; TNF, tumor necrosis factor; RANTES, regulated on activation; normal T cell expressed and secreted; MCP-1, monocyte chemotactic protein-1.

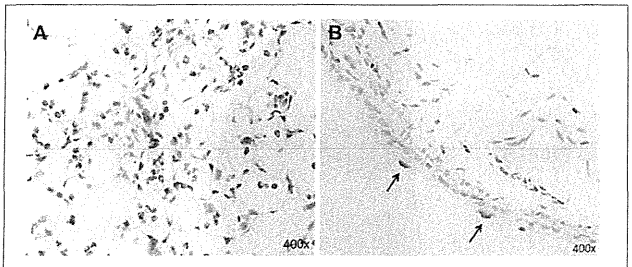


FIGURE 6 | Immunohistochemical staining with RANTES at 8 h and 48 h post-IT challenge. (A) AMs secreted RANTES as early as 8 h after IT challenge; abundant recruited neutrophils in alveolar spaces did not produce RANTES. **(B)** Bronchial epithelial cells positive for RANTES at 48 h post-IT.

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).

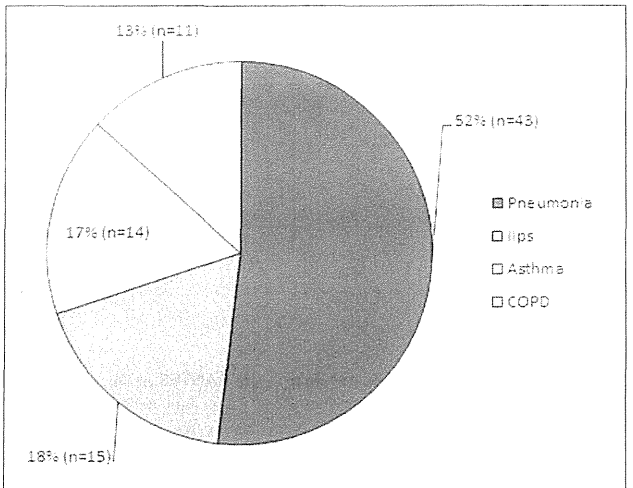


FIGURE 7 | Epidemiological data of adult patients admitted throughout the year to Kyorin University Hospital for respiratory disease. Diagnoses consisted of acute exacerbation of idiopathic interstitial pneumonias ($n = 15$, 18%); pneumonia ($n = 43$, 52%), including 4 cases of definite Mp pneumonia diagnosed by CF antibody titer of ≥ four-fold 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.

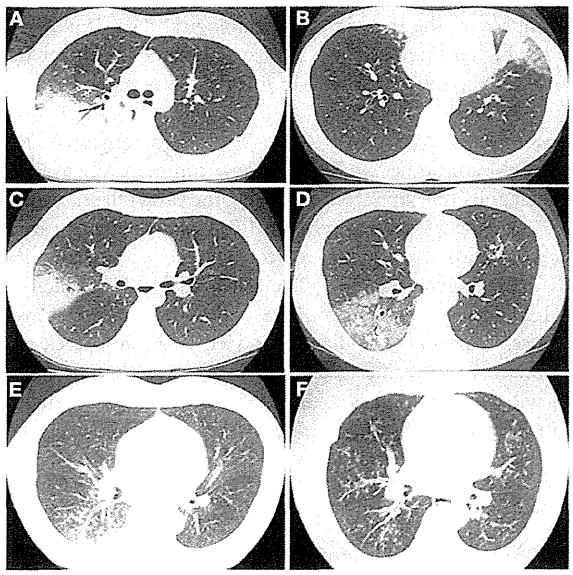


FIGURE 8 | The HRCT findings of Mp pneumonia are characterized as (A) consolidation with air bronchogram surrounded by a crazy paving appearance, (B) consolidation with reticular shadow, (C) consolidation with GGO, (D) GGO with interlobular septal thickening, crazy paving appearance, (E) bronchial wall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

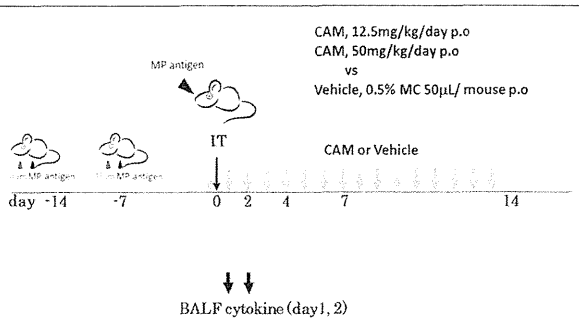


FIGURE 9 | Model E, the clarithromycin (CAM)-treated murine model. Just before intratracheal challenge (IT) with Mp antigen, mice were divided into 3 treatment regimen groups. All were orally treated once a day with CAM (12.5 mg/kg/day or 25 mg/kg/day) or vehicle for 2 consecutive weeks. CAM, clarithromycin;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

([illegible]). *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

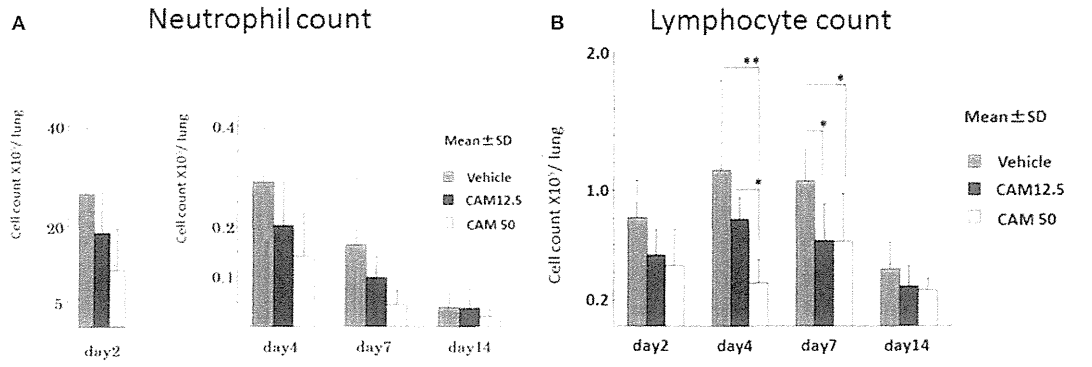


FIGURE 10 | Sequential analysis of bronchial alveolar lavage fluid at days 2, 4, 7, and 14 after intratracheal challenge. (A) No significant difference in the neutrophil count was seen. **(B)** Number of lymphocytes in the CAM-treated groups was significantly suppressed dose-dependently at days 4 and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

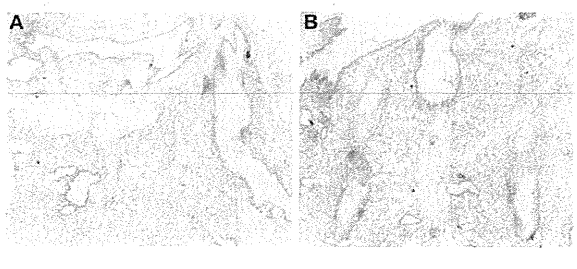


FIGURE 11 | Lymphoplasmacytic infiltration within the PBVA was markedly suppressed in the (A) CAM50 group as compared with the (B) vehicle group. PBVA, peribronchovascular area.

(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