Importantly, angiography does not provide information such as the actual vessel diameter of the lesion or the amount of organized thrombi occupying the lesion. Therefore, intravascular ultrasound (IVUS) is used to evaluate the presence of organized thrombi in the pulmonary arteries and to determine the diameter of the target; subsequently, the balloon is selected depending on the blood vessel diameter as measured by the IVUS (14).

There are some experimental methods to evaluate lesions that are not commonly used. Computed tomography (CT) and/or contrast-enhanced CT have been reported to detect characteristic lesions in CTEPH (28, 29). In one study, cone beam CT was compared with contrast-enhanced CT pulmonary angiography and was found to be useful for the treatment planning of BPA performed distal to the segmental arteries (30). However, not all institutions can perform cone beam CT, and its efficacy has not been compared to that of selective pulmonary angiography. Moreover, the usefulness of optical coherence tomography (OCT) has been reported. The resolution of OCT is high, and OCT allows precise determination of the locations of the target lesions of the pulmonary arteries. It reportedly facilitates the choice of the appropriate balloon size and length (31). Three-dimensional-OCT imaging has been reported to be even more useful in evaluating the lesions (32, 33); however, a downside of this technique is that an image cannot be obtained without having the blood components temporarily eliminated by a jet injection of contrast medium. It sometimes leads to volume overload in severely ill patients with right heart failure. Further, in cases of highly stenotic lesions, the OCT catheter wedges at the lesion and cannot obtain information about lesion beyond the stenosis.

EFFICACY OF BPA

In 2001, Feinstein et al. reported that, with an average of 2.7 BPA procedures, the mean PAP of their patient group decreased by 9 mmHg (12). Similarly, three studies reported from Japan in 2012 demonstrated the effectiveness of BPA, with a mean PAP reduction of 14–21 mmHg from baseline (mean PAP, approximately 45 mmHg) with three to four BPA procedures (14–16); refined BPA demonstrated a larger effect in reducing the mean PAP. We reported to achieve treatment outcomes equivalent to those for PEA, observing significant improvements in the WHO functional class, cardiac index, and pulmonary vascular resistance besides mean PAP (14).

Two studies based on smaller case series focusing on other effects of BPA reported on the right ventricular function measured by echocardiography or cardiovascular magnetic resonance imaging (34, 35); both reports concluded that the right ventricular volume index was improved after BPA. Further, ventilatory inefficiency have also been shown to improve after BPA (18, 36).

In our previous report, at the follow-up catheter examination, significant improvements in the hemodynamics and exercise capacity were maintained (14). Since there have been no reported cases of restenosis after BPA to date, its effectiveness seems to be maintained in the long-term, although only balloon dilatation without stenting is performed. Larger and longer observations and analyses are needed. Moreover, currently, there are insufficient data regarding the long-term survival of patients undergoing BPA. One report stated that the 2-year survival rate after BPA treatment was 100% (15), and in another report, 85% patients were alive after 51 ± 30 months of follow-up (18). Considering the high mean PAP at baseline, these data may offer hope for better survival due to BPA.

MECHANISM OF IMPROVEMENT OF HEMODYNAMICS BY BPA

Pulmonary endarterectomy, the gold standard procedure for treatment of CTEPH, involves removal of intraluminal thrombi from inside the pulmonary arteries; on the other hand, BPA does not remove thrombi from the pulmonary arteries. Therefore, we questioned why and how BPA can lead to improving the hemodynamics without removal of the thrombi. Moreover, we questioned why, unlike for the coronary arteries, restenosis does not occur despite the fact that ballooning alone is performed without using any stents. Previously, we pathologically examined lesions after BPA and found that the only change in the organized thrombi was a small incision (37). A subsequent investigation of an autopsy case revealed that BPA had caused dissection of the tunica media in the treated sites, and that the organized thrombi had partially detached from the vascular wall (**Figure 3**) (38). Thus, this procedure involves the same technique as PEA, only differing in the fact that the thrombi are not extracted from inside the blood vessel, but rather forced to one side to make the lumen larger. Following BPA, parts of the vascular wall exhibited thinning due to dissection; these thin areas of the vascular wall are then subjected to PAP, leading to expansion of the lumen diameter over time without causing restenosis.

COMPLICATIONS OF BPA

COMPLICATION RATE OF BPA

In the first report of BPA, the in-hospital death rate was 5.6% (12), whereas it was slightly lower, at 0–3.4%, in the following reports (14–16). Issues associated with BPA include overcoming lung injury following the procedure. Lung injury was previously thought to be caused by reperfusion edema (12), which is also an important postoperative complication of PEA. In severe cases, mechanical ventilation and percutaneous cardiopulmonary support are required, and the lung injury may be fatal. Although BPA could be considered minimally invasive, BPA-related lung injury reportedly occurs in 9.6–60% of the cases (14, 16, 18). This frequency is higher than in PEA, suggesting that the mechanism causing lung injury in BPA may differ from that in PEA. Cytokine release or a sudden increase in blood flow to the peripheral arteries has been suspected to be the cause of lung injury after PEA (39), and these mechanisms may also be involved in inducing lung injury in BPA.

However, in the case of BPA, vascular injury caused by the guidewire or guiding catheter, or injecting contrast medium with high pressure may also play a role in inducing lung injury. Indeed, by reviewing CT images after BPA and pulmonary angiography, consolidation was observed in the area where BPA was performed in a previous study, and this could also explain why there may be a learning curve for reducing BPA-related lung injury (14). The complication rate for each lesion varies among the lesion types, similar to the fact that the success rate varies depending on the lesion types. In cases of ring-like stenosis and web and abrupt

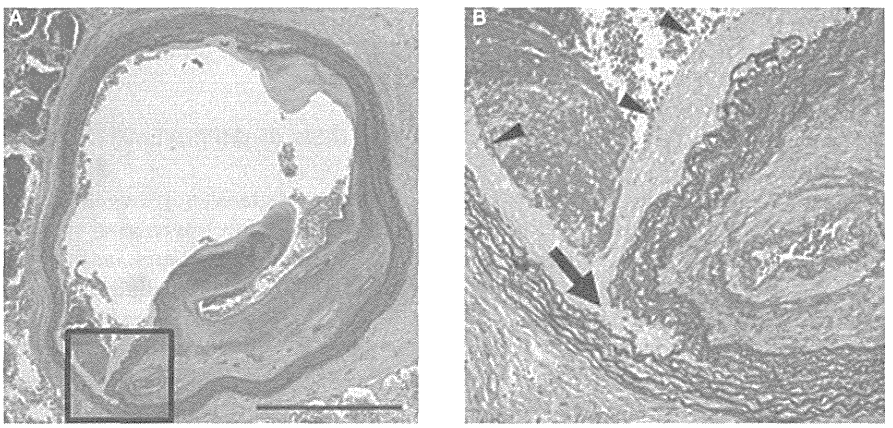

FIGURE 3 | Histology of a pulmonary artery treated by balloon pulmonary angioplasty (BPA) [cited from Ref. (38)]. **(A)** A large lumen was formed due to dissection in the media of a pulmonary artery by BPA (elastic tissue stain, low magnification; bar: 1 mm). **(B)** High magnification of the dissection site [square in **(A)**; elastic tissue stain]. Dissection occurred in the middle of the media (arrow). Newly formed intima is observed on the inner surface (arrowheads). One of the recanalized lumina in the organized thrombi is also seen on the right.

narrowing, a complication rate is 2%. The complication rate is approximately 16% in cases of the subtotal obstruction type, and 10% in pouching defects (23).

In addition, after establishing that BPA is actually dissecting arterial wall (38), some parts of BPA-related lung injury have been found to occur at the dilation site, resulting in overdilatation or progression of dissection of the pulmonary arteries. In addition, even if no signs of complications are observed immediately after BPA, expansion of the thin vessel walls caused by the detachment of organized thrombi may progress in cases with extremely high PAPs, which in turn can lead to oozing ruptures, as observed in aortic dissection or evident pulmonary hemorrhages. There is also a risk of pulmonary artery perforation or vessel rupture with a guide wire (14). It may require emergent transcatheter coil embolization or the use of covered stents (40).

HOW TO REDUCE THE RISK OF LUNG INJURY

Since lung injury was initially thought to be caused by the same mechanism as that occurring after PEA, we attempted administering epoprostenol to lower the PAP and using methylprednisolone and non-invasive positive-pressure ventilation to prevent pulmonary edema (14). However, our findings suggested that these measures could not prevent BPA-related lung injury. Later, an attempt to predict the risk of pulmonary edema resulting from BPA was made by using pulmonary angiography (27). However, if one of the major causes for lung injury is catheter-related, as described above, it might be possible to reduce the risk purely by refining the technique used. For example, using a guidewire with a tip load as small as possible, stopping the tip of the guidewire within the angiographically visible range of the distal vessels, and injecting the contrast agent gently, may all help reduce the risk of lung injury. However, these measures alone are likely not enough, as the complication after BPA has not been eliminated. Further, when lesions have a large amount of organized thrombi, balloon dilation itself may cause excessive extension of the pulmonary vascular wall, likely resulting in vascular injury. Maximum dilatation is considered to be related to more extensive injuries to the vascular wall of the lesion. In order to reduce the risk of vascular wall injuries, the dilatation of the lesion site has to be kept to a minimum. Feinstein et al. reported that high PAP prior to BPA led to a high frequency of postoperative lung injury after BPA (12). Thus, BPA must be performed with particular caution in severe cases.

When keeping the dilatation of the lesion site small, the effectiveness of BPA decreases. The therapeutic effect of BPA is directly proportional to the number of vessels treated (14); thus, even if the dilatation of the lesion sites is kept small, the therapeutic effect of one session can be maintained by treating several lesions in one session. However, if the operator has insufficient experience, treating many lesions at one time leads to an increased risk of vascular injury at many lesions. Thus, the most realistic countermeasure seems to be increasing the number of BPA repetitions.

PRESENT LIMITATIONS OF BPA AND FUTURE DIRECTIONS

There is a need for further understanding and information regarding BPA. A novel classification of BPA suitability according to the lesion type is needed to better select patients who would be most benefited from BPA. Improved strategies to overcome the complications associated with BPA must be established. BPA-specific improvements to devices, such as guiding catheters, guidewires, and balloon catheters, are required. Randomized control trials to evaluate the superiority of BPA over drug therapy and studies on the cost-effectiveness of BPA procedures are needed. Furthermore, long-term data on the risks of restenosis are lacking, as are data on the need for stenting in these patients. Long-term survival and efficacy need to be clarified.

There are also demands for BPA performed in adult patients with isolated peripheral pulmonary artery stenosis (41), and in such cases, there might be a need for stenting (42). We also do not know how to treat very distal arteries with a diameter <100 μm, which can currently not be treated either by PEA or BPA (37).

CONCLUSION

Balloon pulmonary angioplasty is a novel treatment that can potentially provide marked improvements in subjective symptoms and hemodynamics in CTEPH patients ineligible for PEA. However, further refinements of the strategy to reduce complications, improvements in the simplicity of the treatment, and evaluation of the long-term follow-up results are needed before BPA can be recommended as an established treatment for CTEPH.

AUTHOR CONTRIBUTIONS

AO performed the conception of the work, the acquisition, and interpretation of the data for the work and manuscript writing. HM performed the conception of the work and manuscript writing.

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Review

Treatment of idiopathic/hereditary pulmonary arterial hypertension

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ABSTRACT

Treatment of pulmonary hypertension has progressed by recently developed pulmonary arterial hypertension-targeted drugs. However, long-term survival of the patients with idiopathic/heritable pulmonary arterial hypertension is still suboptimal. To improve the outcomes, treatment goals of pulmonary hypertension were proposed at the 5th World Symposium on Pulmonary Hypertension held at Nice, France in 2013; parameters were obtained from cardiopulmonary exercise test, blood tests, echocardiography, and magnetic resonance imaging. In particular, parameters evaluating right ventricular function have been highlighted because survival of the patients with pulmonary arterial hypertension is closely related to right ventricular function. However, treatment specifically targeted to improve right ventricular function in pulmonary hypertension is not yet established. In this setting, we need to maintain or improve right ventricular function with available vasodilators. In this review, we focus on the following two points: (1) Why can pulmonary arterial hypertension-targeted drugs improve right ventricular function without an apparent decrease in pulmonary artery pressure? (2) Are proposed goals sufficient to improve long-term prognosis of the patients? Further, we will discuss what would be the appropriate goal in treating patients with pulmonary arterial hypertension.

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Introduction

Treatment of pulmonary arterial hypertension (PAH) has dramatically advanced by development of PAH-targeted drugs during the past two decades. At present, drugs targeting three pathways that are critical for pathogenesis and progression of PAH are available; namely, prostacyclin analogs that supply the deficient endogenous prostacyclin, endothelin receptor antagonists that inhibit the up-regulated endothelin pathway, and phosphodiesterase-5 inhibitors that compensate the down-regulated nitric oxide pathway. As a result, survival rates reported in recent registries have improved [1–3] (Fig. 1). In this review, we will focus on idiopathic/hereditary PAH (I/HPAH) especially when discussing patient survival because prognosis varies in other forms

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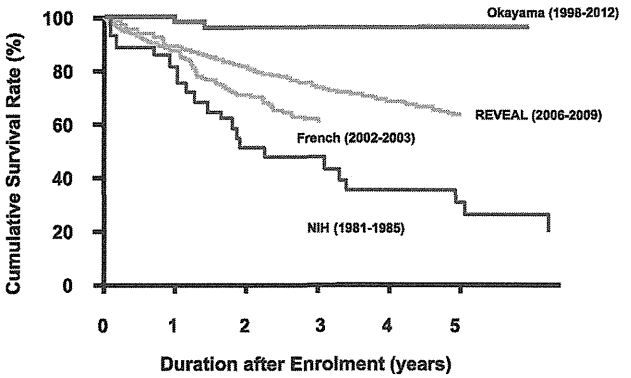

Fig. 1. Kaplan–Meier survival estimates in patients with idiopathic/heritable pulmonary arterial hypertension from reported registries. Red line indicates survival curve from NIH registry [1]. Orange line indicates survival curve from French registry [2]. Green line indicates survival curve from REVEAL registry [3]. Blue line indicates survival curve from Okayama Medical Center [41]. Although cumulative survival of the patients in registry studies has been improving over time, survival rate of the patients at Okayama Medical Center is outstanding. Cited and modified from Refs. [1–3,41].

of PAH in relation to coexisting disorders. In the US National Institutes of Health (NIH) registry conducted during 1981–1985 [1], the 1-, 3-, and 5-year survival rates were 68%, 48%, and 30%, respectively. The outcome was improved in the French registry conducted during 2002–2003 [2] where the 1- and 3-year survival rates were 83% and 58%, respectively. It was further improved in the REVEAL registry conducted during 2006–2009 [3], with 1-, 3-, and 5-year survival rates of 91%, 74%, and 65%, respectively. The treatment algorithm for PAH was updated at the 4th World Symposium on Pulmonary Hypertension (WSPH) held in Dana Point, CA, USA [4]. Worldwide recognition of this evidence-based algorithm may have contributed to the improvement in patient survival. However, PAH remains fatal considering the fact that survival curves show an ongoing decrease over years in the reported registries [2,3]. To improve the long-term survival of the patients with I/HPAH, it is necessary to identify appropriate objective treatment goals for I/HPAH.

Prognostic factors of I/HPAH

It is essential to clarify clinically relevant prognostic factors to identify treatment goals. At the time of the 4th WSPH, the results of the NIH registry provided the only available data [1]. It revealed that the relevant prognostic factors were functional class and hemodynamics. Thus, the goal in treating PAH was set to improve and maintain the patients' functional class at the 4th WSPH [4]. Other recent studies have provided additional prognostic factors besides the functional class and hemodynamic parameters; parameters were obtained from cardiopulmonary exercise test, blood tests, echocardiography, and magnetic resonance imaging as listed in Table 1 [1,5–15]. Among them, parameters representing right ventricular function have come to be emphasized as prognostic indicators at baseline and treatment targets.

Since the leading cause of death in I/HPAH is right ventricular failure [1], it has been recognized that the existence of right ventricular failure would worsen the prognosis of the patients. This recognition was reflected in a previously proposed treatment goal. Although PAH-targeted drugs can decrease pulmonary vascular resistance (PVR) in patients with I/HPAH to some extent (Table 2, [16–22]), none of the drugs are reported to be able to decrease pulmonary artery pressure (PAP) sufficiently. In that case, it is convincing that the patients' prognosis depends on better adaptation of the right ventricle to excessively increased afterload [23].

Table 1
Prognostic predictors in patients with idiopathic/heritable pulmonary arterial hypertension.

Parameter	Values predicting poor survival	Reference
Exercise capacity		
NYHA functional class	III or IV	[1,5,6]
6MWD	<165–307 m	[5,7–9]
Peak VO_2	<10.6–11.6 ml/kg	[8,10]
Hemodynamics		
RAP	>10–20 mmHg	[1,5,9,11]
mean PAP	–	[1]
CI	<2.5 l/min/m^2	[1,6,9]
PVR	>32 Wood units	[5,11]
S_VO_2	<65%	[6]
Biomarkers		
BNP	>50 pg/ml	[5]
NT-proBNP	>1400–1800 ng/l	[6,11]
UA	>6.4 mg/dl (female) >8.9 mg/dl (male)	[10,12]
Respiratory function		
% predicted DLCO	<80%	[1,5]
Echocardiographic measurements		
Right atrial area	–	[13]
Pericardial effusion	Presence	[13]
TAPSE	<1.8 cm	[14]
Magnetic resonance imaging measurements		
SVI	<25 ml/m^2	[15]
RVEDVI	>84 ml/m^2	[15]
LVEDVI	<40 ml/m^2	[15]

NYHA, New York Heart Association functional class; 6MWD, 6-min walk distance; Peak VO_2, peak oxygen consumption; RAP, right atrial pressure; PAP, pulmonary artery pressure; CI, cardiac index; PVR, pulmonary vascular resistance; SvO_2, mixed venous oxygen saturation; BNP, B-type natriuretic peptide; NT-proBNP, N-terminal pro-B-type natriuretic peptide; UA, uric acid; DLCO, carbon monoxide diffusing capacity; TAPSE, tricuspid annular plane systolic excursion; SVI, stroke volume index; RVEDVI, right ventricular end-diastolic volume index; LVEDVI, left ventricular end-diastolic volume index.

Treatment goal recommended in the 5th WSPH

It was timely and meaningful to discuss treatment goals at the 5th WSPH held at Nice, France in 2013. As the committee mentioned, the focus of pulmonary hypertension specialists has shifted from short-term functional changes to improvements in long-term outcomes [24]. The focus of the discussion at the 5th WSPH was to set higher the "bar" of treatment goals. The recommended treatment goals included the following: modified New York Heart Association functional class I or II, 6-min walk distance ≥380–440 m, cardiopulmonary exercise test–measured peak oxygen consumption >15 ml/min/kg, and ventilatory equivalent for carbon dioxide <45 l/min/l/min, B-type natriuretic peptide (BNP) level toward "normal," echocardiography and/or cardiac magnetic resonance imaging demonstrating normal/near-normal right ventricular size and function, and hemodynamics showing normalization of right ventricular function with right atrial pressure <8 mmHg and cardiac index >2.5–3.0 l/min/m^2 [24]. Since right ventricular function came to be considered among prognostic indicators at baseline, maintaining right ventricular function was emphasized as the treatment goal. Then, how can we maintain or improve right ventricular function in PAH?

Established medications to treat chronic left heart failure with decreased ejection fraction, such as angiotensin-converting enzyme inhibitors and beta-blockers, have no evidence to ameliorate right heart failure of I/HPAH. Moreover, Provencher et al. [25] reported that beta-blockers were associated with significant worsening in exercise capacity and pulmonary hemodynamics in patients with moderate to severe PAH. The evidence in

Table 2
Effects of pulmonary hypertension-specific drugs on idiopathic/heritable pulmonary arterial hypertension.

Drug	Reference	Patient numbers[a]	Duration (months)	Drug delivery	Dose	NYHA	PVR (dyn s cm^{-5})			mean PAP (mmHg)		
							Baseline	Effect	p	Baseline	Effect	p
Bosentan	[16]	21 (17)	3	po	250 mg/day	III	896 ± 425	−223	<0.001	54 ± 13	−1.6	0.013
Ambrisentan	[17]	64 (39)	3	po	1–10 mg/day	II–III	840 ± 407	−226	<0.05	49 ± 13	−5.2	<0.05
Sildenafil	[18]	69 (44)	3	po	60 mg/day	II–IV	987 ± 464	−122	0.01	54 ± 13	−2.1	0.04
Tadalafil	[19]	79 (46)	4	po	40 mg/day	I–III	901 ± 488	−209	0.039	54 ± 8	−4.3	0.01
Riociguat	[20]	254 (149)	3	po	7.5 mg/day	I–IV	791 ± 453	−223	<0.001	47 ± 15	−4	<0.001
Treprostinil	[21]	233 (134)	3	sc	9.3 ng/kg/min[b]	II–IV	2078 ± 80[c]	−280[c]	0.0001	62 ± 1	−2.3	0.0003
Epoprostenol	[22]	41 (41)	3	iv	9.2 ± 0.8 ng/kg/min[b]	III–IV	1279 ± 80	−272	<0.002	61 ± 2	−4.8	<0.001

NYHA, New York Heart Association functional class; PVR, pulmonary vascular resistance; PAP, pulmonary artery pressure; po, per os; sc, subcutaneous; iv, intravenous.
[a] Total patients (patients with I/HPAH).
[b] Average dose.
[c] Pulmonary vascular resistance index (dyn s cm^{-5} m^2).

treating left heart failure which is not induced by pressure overload cannot be directly extrapolated in the treatment of right heart failure induced by pressure overload. Digitalis is the only recommended medication as supportive therapy in the updated evidence-based treatment algorithm in PAH [26]; although there is no evidence for long-term effect of digitalis on right heart failure in patients with PAH. The sole currently available method to maintain or improve right heart function in I/HPAH is to use vasodilators approved for treatment of PAH [23].

Effect of vasodilators on right heart function

PVR is calculated by subtracting pulmonary capillary wedge pressure from mean PAP and dividing it by cardiac output. It is a useful measure of right ventricular afterload in understanding the averaged cardiac function per minute. However, it is difficult to understand, why vasodilators decrease PVR mainly by increasing cardiac output.

A pressure–volume diagram of the ventricle would be of help in understanding this phenomenon. Ventricular end-systolic pressure–volume relationship (ESPVR) is theoretically linear, under the physiological condition, as shown in Fig. 2Aa [27]. The slope of ESPVR indicates end-systolic ventricular elastance which is known as the index of ventricular contractility [27]. The afterload of the ventricle on the pressure–volume diagram is indicated by arterial elastance which is derived from end-systolic pressure divided by stroke volume [28]. In this setting, increase in stroke volume and decrease of end-systolic pressure due to the decrease of afterload is always proportional (Fig. 2Ab). However, the actual ESPVR is nonlinear outside the physiological range [29,30]. ESPVR around the extremely high end-systolic pressure shows upward convexity as shown in Fig. 2Ba. Thus, afterload reduction obtained by vasodilators results in increase of stroke volume with only a slight decrease of end-systolic pressure. On the other hand, ESPVR around the low end-systolic pressure shows a steeper slope. Thus, equivalent after-load reduction results in a larger decrease of end-systolic pressure with a small increase in stroke volume. Therefore, the actual relation between end-systolic pressure and stroke volume indicates upward convexity as shown in Fig. 2Bb [31].

The right ventricle of the patients with PAH would be working under this condition. Representative pressure–volume loops of the right ventricle recorded from a patient with IPAH are shown in Fig. 3. Although ESPVR in these loops appears linear, volume axis intercept of extrapolated linear ESPVR is negative. Volume axis intercept of ESPVR should be theoretically positive and hence, ESPVR of these loops would be nonlinear as indicated by the dotted line. In other words, if we could reduce PVR to some extent by vasodilators in patients with PAH who exhibit a high afterload of the right ventricle, increase in right heart pump function (cardiac output) is achievable [32].

Can current therapeutic goals lead to improvement of long-term survival?

The treatment goal recommended at the 5th WSPH [24] might contribute to an improvement in the prognosis of PAH. However, we consider that sufficient improvement in long-term survival of the patients cannot be obtained by the current treatment goal, based on the following three reasons.

Firstly, the evidence for the current treatment goals would have been obtained from the data of the patients treated by aiming at short-term improvement of functional class. Therefore, some of the currently recommended treatment goals appear to be insufficient to achieve long-term survival of the patients. For example, correlation between 6-min walk distance at baseline and patients' survival was observed; whereas the short-term improvement of 6-min walk distance was not correlated with patients' survival in a meta-analysis of 22 randomized trials regarding PAH [33]. The same holds true for the hemodynamic indices. Right atrial pressure <8 mmHg and cardiac index >2.5 l/min/m^2 were already achieved in previous studies regarding epoprostenol monotherapy. According to the report in 2002 from France, cardiac index was improved to 2.5 l/min/m^2 in adult cases treated with epoprostenol alone [34]. The 1-, 3-, and 5-year survival rates in the report were 85%, 63%, and 55%, respectively. In pediatric cases treated with epoprostenol alone reported in 2004 from the USA [35], right atrial pressure and cardiac index were improved to 6 mmHg and 4.5 l/min/m^2 with 1-, 3-, and 5-year survival rates of 83%, 66%, and 57%, respectively. Although goals of hemodynamic parameters were already achieved, the prognosis was not good enough. Further, although these results were reported more than 10 years ago when there were fewer treatment options, outcomes reported in recent registry studies have not much improved [3,34,35] (Fig. 4).

Secondly, it seems impossible to normalize the size of the dilated right ventricle even when all other recommended goals are achieved. Right ventricular size usually gets smaller after initiation of treatment. However, even after hemodynamic improvement is achieved, the size of the right ventricle cannot be normalized. As described above, increase of cardiac output has been achieved in many previous studies using PAH-targeted drugs. However, there is no study reporting significant regression of right ventricular dilatation with current medications. A previous study has merely demonstrated that better prognosis could be expected in patients with normal/near-normal right ventricular size at baseline [5]. Since it has not been demonstrated that improvement of right ventricular dilatation can be achieved by successful treatment,

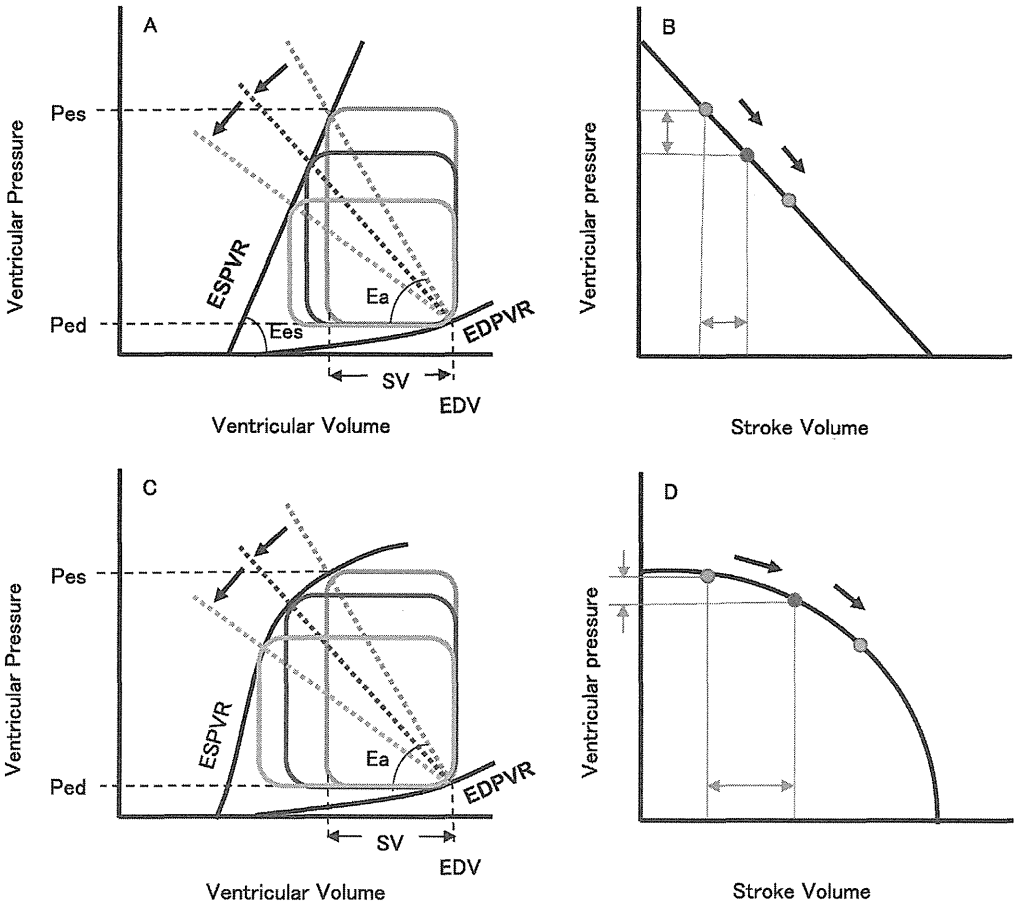

Fig. 2. Schematic illustrations of pressure–volume relation of the ventricle (panel a) and ventricular pump function graph (panel b). (A) Schematic illustrations when assumed that end-systolic pressure–volume relation (ESPVR) of the ventricle is always linear. (a) The slope of the line drawn from end-diastolic pressure–volume point to end-systolic pressure–volume point in the pressure–volume loop represents arterial elastance (Ea). Ea is a measure of ventricular afterload in pressure–volume relation. When the contractility of the ventricle is unchanged (Ees is unchanged), decrease of Ea from green dotted line to red dotted line results in increase of stroke volume (SV) and decrease of end-systolic pressure (Pes). Additional equivalent decrease of Ea from red dotted line to blue dotted line results in the same amount of increase of SV and decrease of Pes when ESPVR is linear. (b) Increase in SV and decrease of Pes due to the decrease of afterload is always proportional when ESPVR is linear and thus, corresponding points to green, red and blue loops in panel Aa indicate linear relation. (B) Schematic illustrations under actual nonlinear ESPVR of the ventricle. (a) When pressure is high, ESPVR is not linear. In this case, decrease of Ea from green dotted line to red dotted line results in increase of SV with a slight decrease of Pes. Additional equivalent decrease of Ea from red dotted line to blue dotted line results in smaller increase in SV and larger decrease in Pes because ESPVR is near-linear. (b) Corresponding points to green, red, and blue loops in panel Ba indicate nonlinear relation. EDPVR, end-diastolic pressure–volume relation; EDV, end-diastolic volume; Ees, end-systolic elastance of the ventricle; Ped, end-diastolic pressure.

there is a leap in logic to state that normalization of the right ventricle should be aimed at.

Thirdly, it might be difficult to achieve the goals with the currently recommended treatment algorithm. It recommends epoprostenol only for patients with NYHA functional class IV [26]. It is also stated that if the clinical response is inadequate, combination therapy should be considered. These statements have not changed from the previously proposed treatment algorithm at the 4th WSPH [4]. However, in the current situation, the augmentation of medical therapy, including initiation of add-on therapy and switching to intravenous epoprostenol therapy, appears to be performed inappropriately. For example, in the French registry, only 10.6% of the patients were treated with combination therapy and 15.4% of the patients were treated with intravenous epoprostenol, although 29% of the patients had died during 3 years follow-up [2]. In the REVEAL registry, only 47.6% of the patients with functional class IV were treated with intravenous or subcutaneous prostacyclin [36]. This tendency can be partly explained by the patients' and physicians' hesitation toward proceeding to the most aggressive and complicated treatment option, intravenous prostacyclin. We consider that ambiguous definition of "adequate clinical response" is also a contributing factor of delay for initiation of appropriate treatment. Many physicians might consider the meaning of "adequate clinical response" as maintaining the condition instead of improving it.

To improve the truly long-term prognosis

The essential pathophysiological feature of PAH is increased PVR caused by remodeling of pulmonary arteries. Right heart failure is merely the consequence of sustained overload. On the other hand, the major cause of death in I/HPAH is right ventricular failure; and thus, we agree with the opinion that the prevention of right heart failure will improve the long-term prognosis of the patients. However, it is difficult to avoid right ventricular failure in patients with PAH with high PAP. Right ventricular adaptation progresses in response to increased wall stress due to increased pressure [15,23]. Pulmonary arterial remodeling would also be accelerated in response to increased vascular wall stress [37].

There are case reports that demonstrated persistence of normalized PAP after complete abolition of the allograft in patients who underwent single-lung transplantation for the treatment of

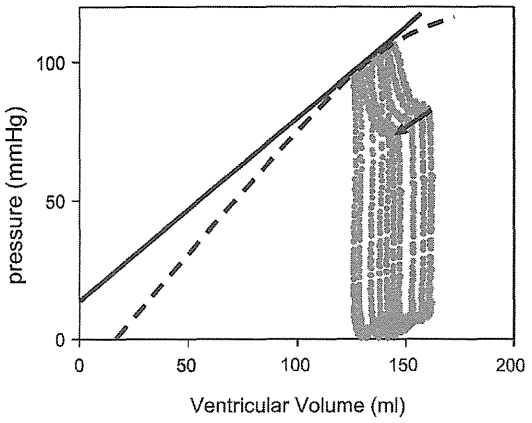

Fig. 3. Representative pressure–volume loops of the right ventricle recorded from a patient with idiopathic pulmonary arterial hypertension (green loops). Preload reduction by partial occlusion of inferior vena cava shifted the loop to leftward and downward (indicated by arrow). Although end-systolic pressure–volume relation (ESPVR) in these loops appears linear, volume axis intercept of extrapolated linear ESPVR (solid line) is negative. Volume axis intercept of ESPVR should be theoretically positive; and hence, ESPVR of these loops would be nonlinear as indicated by dotted line.

IPAH [38,39]. These patients had originally been in a severe condition with PAH requiring lung transplantation and could survive with their single native lung for years. Their PAP was kept at near normal level even after chronic rejection of allograft. The fact suggests that vascular remodeling in their native lung would have regressed. It is particularly of note that hemodynamically- [38] or pathologically- [39] confirmed regression of established pulmonary arterial remodeling could be achieved by reducing pressure and flow with single-lung transplantation. These cases indicate that stress of pressure and/or flow would play a role in precipitating the disease process in some patients with PAH.

Persistent high PAP and wall stress will further progress the pulmonary artery remodeling, leading to further increase in PVR. As a result, right ventricular remodeling will progress and lead to maladaptation of the right ventricle. In order to stop or reverse remodeling of the right ventricle, normalization of right ventricular wall stress should be necessary. In other words, to obtain the long-term survival of the patients, it is necessary to achieve sufficient decrease of PAP. Needless to say, decrease in PAP discussed here does not mean the result of deterioration of right heart failure. It should be the result of sufficient afterload reduction of the right ventricle obtained by appropriate treatment. It needs to be achieved by decreasing pulmonary arterial elastance without decreasing right ventricular elastance (Fig. 2Ba). In patients with aortic stenosis, leaving stenotic valve and optimizing medical treatment of left heart failure expecting the left ventricular adaptation cannot improve the long-term survival of the patients. The same would be true for patients with I/HPAH. The primary cause of right heart failure in I/HPAH is excessive pressure overload. It is natural to aim at sufficient reduction of afterload to achieve the long-term survival of the patients.

Is it possible to decrease PAP in I/HPAH?

It is necessary to sufficiently decrease PAP, which can ultimately stop the progression of right ventricular remodeling, in order to achieve the long-term survival of the patients with I/HPAH. Nevertheless the amount of reduction of PAP has not been questioned at all in the treatment of I/HPAH in previous WSPHs. Or rather, it has been believed that I/HPAH is a progressive disease in nature and substantial decrease of PAP cannot be obtained by approved PAH-targeted drugs in patients with established I/HPAH. Indeed, according to the recent studies, decrease of PAP has not been achieved at all. For example, treatment effect in PVR was a 2.2% decrease and PAP did not decrease at all in a recent registry [6].

In the early 1990s, there were no approved PAH-targeted drugs other than epoprostenol. Physicians did not hesitate to use epoprostenol for patients who needed it, and they aggressively up-titrated the dosage of epoprostenol to obtain the maximal effect. In previous reports of epoprostenol monotherapy for adult and pediatric patients with I/HPAH, PAP decreased by 8 mmHg and 19 mmHg, with decreases of 33% and 54% in PVR [34,35]. More than 10 years ago, sufficient decrease of PVR along with decrease of PAP was indeed achieved. Failure in decreasing PAP in recent studies might be a result of the treatment aiming at short-term improvement of functional class. We should be able to reduce PAP with several therapeutic options if we could use them appropriately, with appropriate timing.

What would be the true "goal" in treating patients with I/HPAH?

The definition of goal should be reconsidered. The "goal" of treatment is ideally to cure the disease. The goals stated in the present treatment algorithm are parameters to be aimed at and criteria to be cleared, to achieve the true "goal". Since the currently

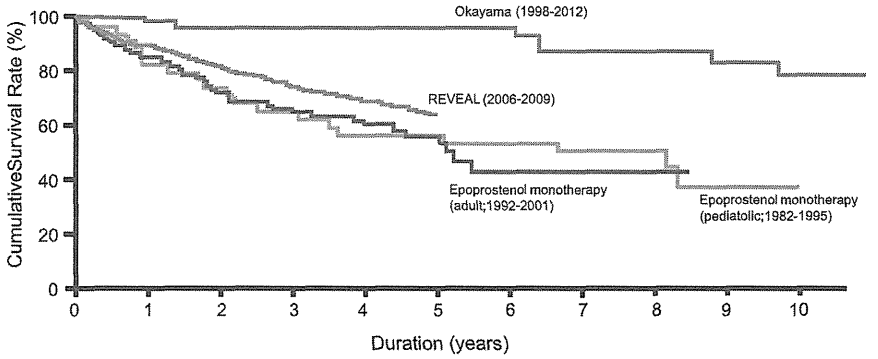

Fig. 4. Reported Kaplan–Meier survival estimates in patients with idiopathic/heritable pulmonary arterial hypertension treated with epoprostenol. Red line indicates survival curve of adult patients from France [34]. Orange line indicates survival curve of pediatric patients from USA [35]. Although, these outcomes were reported more than 10 years ago, there is not much difference between these survival curves and survival curves from REVEAL registry (green line) [3]. Compared to these data, survival rate reported from Okayama (blue line) [41] is outstanding.
Cited and modified from Refs. [3,34,35,41].

Fig. 5. Survival rate of the patients treated at Okayama Medical Center, stratified by mean PAP at follow-up [41]. Survival rate of the patients with mean PAP ≥ 42.5 mmHg was significantly worse than patients with mean PAP < 42.5 mmHg ($p < 0.01$). (m)PAP, (mean) pulmonary artery pressure.
Cited and modified from Ref. [41].

available data show that it is difficult to cure PAH, we should at least aim at long-term survival of the patients for more than 10 years. Then, we need to think what criteria should be fulfilled to obtain better prognosis. In the recommendation at the 5th WSPH, it is not shown how many criteria should be achieved, which criteria should be at least fulfilled, and in what time period criteria should be cleared.

We had aimed at treating patients with I/HPAH medically as a bridge to lung transplantation. However, in 2006, we observed PAP decreased with preserved cardiac output in some patients with I/HPAH. At that time, we realized it is possible to reduce PAP in patients with I/HPAH and shifted the treatment "goal" to aiming at improvement of 10-year survival. To achieve our new "goal", we considered it would be necessary to reduce PAP while maintaining cardiac output. To be specific, we set the criteria of mean PAP as <40 mmHg, based on the results of calcium channel blocker responders who were already reported to be able to achieve 5-year survival of 94% [40].

In our cohort of 56 patients with I/HPAH treated from 1998 to 2012 [41], we demonstrated a better prognosis compared to any other previous reports. PVR was reduced by 67% and PAP was decreased by 44% with treatment. Although only about a half of the patients could achieve mean PAP < 40 mmHg; mean survival time from the diagnosis was 14.9 ± 0.8 years (95% CI, 13.4–16.4 years), with 1-, 3-, 5-, and 10-year survival rates of 98%, 96%, 96%, and 78%, respectively. Mean PAP was an important predictor of prognosis and all the patients who could achieve mean PAP < 42.5 mmHg survived during the study period (Fig. 5). Among the patients who could not achieve mean PAP < 42.5 mmHg, 5-year survival rate was as high as ~80% whereas 10-year survival rate was as low as ~50%. These results suggest that to improve long-term survival of the patients with I/HPAH, criteria to be achieved should be a decrease of afterload which leads to reduction of PAP (mean PAP < 42.5 mmHg). Needless to say, this huge reduction of afterload cannot be accomplished by treating with any single drug. Combination therapy with different modes of action should be necessary. In fact, in survivors of our cohort, 78% of the patients were on combination therapy and epoprostenol was prescribed in 76% of the patients.

Our cohort included Japanese patients only and it might not be possible to apply these results to other ethnicities. However, since sufficient reduction of PVR has already been reported to be possible with epoprostenol in western countries in the past, it is expected to be possible in other countries if treatment were pursued appropriately. Although there might be difficulties in obtaining consent from the patients and covering high medical costs, we should utilize all the available treatment options for better survival. We do not know whether our criteria are sufficient in targeting 15- or 20-year survival. It is still to be investigated whether there are better criteria to fulfill rather than to improve pulmonary hemodynamics.

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MAJOR ARTICLE

Mogamulizumab, an Anti-CCR4 Antibody, Targets Human T-Lymphotropic Virus Type 1–infected $CD8^+$ and $CD4^+$ T Cells to Treat Associated Myelopathy

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Background. Human T-lymphotropic virus type 1 (HTLV-1) can cause chronic spinal cord inflammation, known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). Since $CD4^+CCR4^+$ T cells are the main HTLV-1 reservoir, we evaluated the defucosylated humanized anti-CCR4 antibody mogamulizumab as a treatment for HAM/TSP.

Methods. We assessed the effects of mogamulizumab on peripheral blood mononuclear cells from 11 patients with HAM/TSP. We also studied how $CD8^+$ T cells, namely $CD8^+$ $CCR4^+$ T cells and cytotoxic T lymphocytes, are involved in HTLV-1 infection and HAM/TSP pathogenesis and how they would be affected by mogamulizumab.

Results. Mogamulizumab effectively reduced the HTLV-1 proviral load (56.4% mean reduction at a minimum effective concentration of 0.01 μg/mL), spontaneous proliferation, and production of proinflammatory cytokines, including interferon γ (IFN-γ). Like $CD4^+CCR4^+$ T cells, $CD8^+CCR4^+$ T cells from patients with HAM/TSP exhibited high proviral loads and spontaneous IFN-γ production, unlike their $CCR4^-$ counterparts. $CD8^+CCR4^+$ T cells from patients with HAM/TSP contained more IFN-γ–expressing cells and fewer interleukin 4–expressing cells than those from healthy donors. Notably, Tax-specific cytotoxic T lymphocytes that may help control the HTLV-1 infection were overwhelmingly $CCR4^-$.

Conclusions. We determined that $CD8^+CCR4^+$ T cells and $CD4^+CCR4^+$ T cells are prime therapeutic targets for treating HAM/TSP and propose mogamulizumab as a new treatment.

Keywords. HTLV-1; HAM/TSP; CCR4; mogamulizumab; CD8.

Human T-lymphotropic virus type 1 (HTLV-1) infects 10–20 million people worldwide, causing HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia/lymphoma (ATL) in a small fraction of infected individuals [1–3]. HAM/TSP is an inflammatory disease of the central nervous system (CNS) that is thought to develop via so-called bystander damage, meaning that the host immune responses to HTLV-1–infected cells in the CNS damage the spinal cord [4]. Currently established treatments for HAM/TSP, such as corticosteroids [5] and interferon alfa [6], do not effectively reduce the HTLV-1 proviral load, which is well correlated with disease severity [7]. Reverse transcriptase inhibitors, which are used against human immunodeficiency virus type 1, were not effective against HTLV-1 in

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clinical trials [8, 9]. These and other existing antiviral drugs usually block the viral replication process [10], but HTLV-1 may escape these drugs by replicating using host cell division [11, 12]. The ideal treatment strategy for HAM/TSP would be selectively targeting and eliminating the HTLV-1–infected cells, but no such treatments currently exist.

Mogamulizumab, a defucosylated humanized anti-CCR4 immunoglobulin G1 (IgG1) monoclonal antibody, was recently approved in Japan as a novel therapy for ATL [13]. Importantly, ATL cells usually express chemokine receptor CCR4 [14]. Mogamulizumab strongly binds to Fcγ receptor IIIa (FcγRIIIa) on natural killer (NK) cells and elicits powerful antibody-dependent cellular cytotoxicity (ADCC) against the $CCR4^+$ ATL cells [15, 16].

Recently, we found that $CD4^+CD25^+CCR4^+$ T cells are the main HTLV-1 reservoir in HAM/TSP [17]. These cells abnormally produce interferon γ (IFN-γ) and are thought to play an important role in producing the chronic inflammation in HAM/TSP [17]. Thus, we began investigating the possibility of treating HAM/TSP and ATL by targeting $CCR4^+$ cells. We have already established that the defucosylated human/mouse chimeric anti-CCR4 antibody KM2760 effectively reduces the HTLV-1 proviral load in cultured peripheral blood mononuclear cells (PBMCs) from patients with HAM/TSP [18]. Here, we evaluate for the first time the effects of the humanized antibody mogamulizumab on cells from patients with HAM/TSP.

There is a population of $CD8^+$ T cells that express CCR4, but these cells have so far received much less attention than $CD4^+$ $CCR4^+$ T cells from HTLV-1 researchers. Although it has been shown that HTLV-1 infects $CD8^+$ T cells [19], it is as of yet unknown which among $CD8^+$ T cells are predominantly infected, as well as whether and how the infection influences the functions of those cells. $CD8^+CCR4^+$ T cells are reported to suppress inflammation and play a beneficial role in controlling chronic inflammatory diseases [20, 21]. It is important to determine whether $CD8^+CCR4^+$ T cells are protective or harmful during HAM/TSP pathogenesis, as well as how these cells would be affected by mogamulizumab.

We hypothesized that $CCR4^+$ cells among $CD8^+$ and $CD4^+$ T cells are highly infected and liable to develop proinflammatory traits. It has been reported that HTLV-1 preferentially transmits to $CCR4^+$ T cells through CCL22 (a CCR4 ligand) production induced by the HTLV-1 protein product Tax [22]. Tax has also been reported to induce IFN-γ production via transcriptional alterations within the infected cells themselves [18].

In the present study, we determined that mogamulizumab is effective at reducing the proviral load and proinflammatory character in PBMCs from patients with HAM/TSP. Next, we revealed that $CD8^+CCR4^+$ T cells are indeed highly infected by HTLV-1 and become proinflammatory. Finally, we determined that the majority of Tax-specific cytotoxic T lymphocytes (CTLs) were $CCR4^-$, indicating that they would not be inadvertently targeted by mogamulizumab. Our results indicate that $CD8^+CCR4^+$ T cells should be considered a key therapeutic target when developing treatments for HAM/TSP and that mogamulizumab represents a viable candidate for such a treatment.

METHODS

Subjects

This study was approved by the Institutional Ethics Committee at St. Marianna University and conducted in compliance with the Declaration of Helsinki. All participants gave written informed consent. Blood samples were obtained from 11 patients with HAM/TSP (8 females and 3 males; median age, 57 years [range, 47–72 years]; proviral load, 4.7 copies/100 cells [range, 1.26–9.71 copies/100 cells]), 8 HTLV-1–positive asymptomatic carriers (6 females and 2 males; median age, 57 years [range, 28–76 years]; proviral load, 4.7 copies/100 cells [range, 2.43–13.19 copies/100 cells]), and 8 HTLV-1–negative healthy volunteers (6 females and 2 males; median age, 59 years [range, 51–72 years]). HTLV-1 seropositivity was determined by a particle agglutination assay (Fujirebio, Tokyo, Japan) and confirmed by Western blot (SRL Inc., Tokyo, Japan). HAM/TSP was diagnosed according to the World Health Organization guidelines [23]. PBMCs were separated by Ficoll-Hypaque density gradient centrifugation (Pancoll; PAN-Biotech, Aidenbach, Germany) and viably cryopreserved in liquid nitrogen with freezing medium (Cell Banker 1; Mitsubishi Chemical Medience Corporation, Tokyo, Japan).

Cell Culture

PBMCs were seeded at 1×10^5 cells/200 μL/well in 96-well round-bottomed plates in the presence or absence of mogamulizumab or KM2760 (gifts from Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) or human control IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or prednisolone (LKT Laboratories, Inc., St. Paul, MN) and incubated at 37°C in 5% CO_2. Roswell Park Memorial Institute 1640 medium was supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin antibiotic solution (Wako Pure Chemical Industries Ltd., Osaka, Japan). The supernatants were collected and stored at −80°C. The cells were harvested for DNA extraction or fluorescence-activated cell-sorter (FACS) analysis. The HTLV-1 proviral load was measured using ABI Prism 7500 SDS (Applied Biosystems, Carlsbad, CA), as described previously [24].

Cell Proliferation Assay

PBMCs from patients with HAM/TSP were cultured for 7 days as described above. PBMCs from healthy donors were stimulated with 4 μg/mL of phytohemagglutinin-P (PHA; Sigma-Aldrich, St. Louis, MO) and cultured in the presence or absence of mogamulizumab or prednisolone for 3 days. During the last 16 hours, 1 μCi of ^{3}H-thymidine was added to each well, and then cells were harvested and counted with a β-plate counter (Wallac-Perkin Elmer, Waltham, MA). The assay was performed in triplicate, and average values were used for analysis.

Measurement of Cytokines

The concentrations of 6 cytokines (IFN-γ, interleukin 2 [IL-2], interleukin 4 [IL-4], interleukin 6 [IL-6], interleukin 10 [IL-10], and tumor necrosis factor α [TNF-α]) in culture supernatants were measured with a cytometric bead array kit (BD Biosciences, San Diego, CA), using a FACSCalibur flow cytometer (BD Biosciences).

Flow Cytometric Analysis

Cells were immunostained with various combinations of the following fluorescence-conjugated antibodies to surface antigens: anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD14 (61D3), and anti-CD19 (HIB19), from eBiosciences (San Diego, CA); and anti-CD56 (B159) anti-CCR4 (1G1), from BD Biosciences. The epitope of the anti-CCR4 antibody (1G1) is different from that of mogamulizumab and KM2760, and thus these treatments do not affect the binding of 1G1 to CCR4 [16]. In some experiments, allophycocyanin-conjugated HLA-A*2402/HTLV-1 Tax301-309 tetramer (Medical & Biological Laboratories, Nagoya, Japan) was used. To identify HTLV-1–infected cells, cells were fixed and permeabilized using a staining buffer set (eBiosciences) and then intracellularly stained with anti-Tax antibody (Lt-4) [25]. To analyze intracellular effector molecules, cells were fixed and stained with the antibodies to perforin (δG9) and granzyme B (GB11; BD Biosciences). For intracellular cytokine staining, PBMCs were stimulated for 6 hours with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL, Sigma-Aldrich Japan, Tokyo, Japan) in the presence of monensin (GolgiStop, BD Biosciences). After being harvested, the cells were fixed and stained with the antibodies to IFN-γ (B27) and IL-4 (MP4-25D2; BD Biosciences). The stained cells were analyzed using FACSCalibur, and the data were processed using FlowJo software (TreeStar, San Diego, CA). For cell sorting, $CD8^+$ T cells were negatively selected from PBMCs, using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified $CD8^+$ T cells were stained with anti-CD3, anti-CD8, and anti-CCR4 antibodies, and then $CD3^+CD8^+$, $CD3^+CD8^+CCR4^+$, and $CD3^+CD8^+CCR4^-$ T cells were separated using a cell sorter (JSAN, Bay Bioscience Co., Ltd., Hyogo, Japan). The purity exceeded 95%.

Statistical Analysis

Values are expressed as means ± standard deviations. The paired *t* test or the Wilcoxon signed-rank test was used for within-group comparisons. The Mann–Whitney *U* test was used for comparisons between groups. Repeated-measures analysis of variance (ANOVA) followed by the Dunnett test or the Friedman test followed by the Dunn test were used for paired multiple comparisons. Statistical analyses were performed using GraphPad Prism 5 and Prism statistics (GraphPad Software, Inc., La Jolla, CA), and *P* values of <.05 were considered statistically significant.

RESULTS

Mogamulizumab and KM2760 Reduce the HTLV-1 Proviral Load and Inhibit Spontaneous Proliferation of PBMCs From Patients With HAM/TSP

The effects of mogamulizumab and KM2760 were assessed by measuring proviral loads in treated PBMCs from patients with HAM/TSP. ^{3}H-thymidine incorporation was used to assess the inhibitory effects of the antibodies on spontaneous cell proliferation, a distinctive phenomenon associated with PBMCs from HTLV-1–infected individuals by which the cells proliferate without mitogens or stimuli in vitro [26]. Mogamulizumab and KM2760 both reduced proviral load in a dose-dependent manner at concentrations of ≥0.01 μg/mL (mean reduction [±SD], 56.4% ± 21.1% and 61.1% ± 17.0%, respectively; $P < .01$ and $P < .001$, respectively; Figure 1*A*). Notably, there was a mean reduction (±SD) of 66.4% ± 20.2% in the proviral load with 10 μg/mL mogamulizumab ($P < .001$), which is the blood concentration of the antibody in patients with ATL treated with 1 mg/kg mogamulizumab [13]. Mogamulizumab and KM2760 similarly inhibited spontaneous proliferation in a dose-dependent manner at concentrations of ≥0.01 μg/mL (mean inhibition [±SD], 25.6% ± 31.9% and 22.1% ± 35.9%, respectively; $P < .01$ and $P < .05$, respectively; Figure 1*B*). Because mogamulizumab and KM2760 showed such similar results, only mogamulizumab was used in the next experiments as representative of the 2. Mogamulizumab was also tested against human IgG to control for nonspecific antibody effects and more effectively reduced the proviral load and spontaneous proliferation (Supplementary Figure 1*A* and 1*B*). Mogamulizumab reduced the HTLV-1 proviral load in cells from asymptomatic carriers, as well, in a dose-dependent manner (Figure 1*C*). Finally, mogamulizumab inhibited PHA-stimulated proliferation of PBMCs from healthy donors at concentrations of ≥0.1 μg/mL ($P < .01$), but the effects of prednisolone were much more pronounced than those of mogamulizumab (prednisolone vs mogamulizumab, $P < .001$; Figure 1*D*).

Prednisolone suppressed spontaneous proliferation (mean inhibition [±SD], 37.4% ± 35.2%; $P < .001$; Figure 1*B*) but did not decrease proviral load (Figure 1*A*). The combination of mogamulizumab and 0.1 μg/mL of prednisolone, which corresponds to the serum concentration achieved when 5 mg of prednisolone is administered orally [27], reduced proviral load as much as but no more than did mogamulizumab alone (Supplementary Figure 2*A*). On the other hand, ^{3}H-thymidine incorporation was substantially more inhibited by the combination treatment than with mogamulizumab alone (mean inhibition [±SD], 81.3% ± 18.3% vs 71.3% ± 19.3%; $P = .01$; Supplementary Figure 2*B*).

Mogamulizumab and KM2760 Inhibit Proinflammatory Cytokine Production in PBMCs From HAM/TSP Patients

Here we examined the effects of mogamulizumab and KM2760 on cytokine production in PBMCs from patients with HAM/

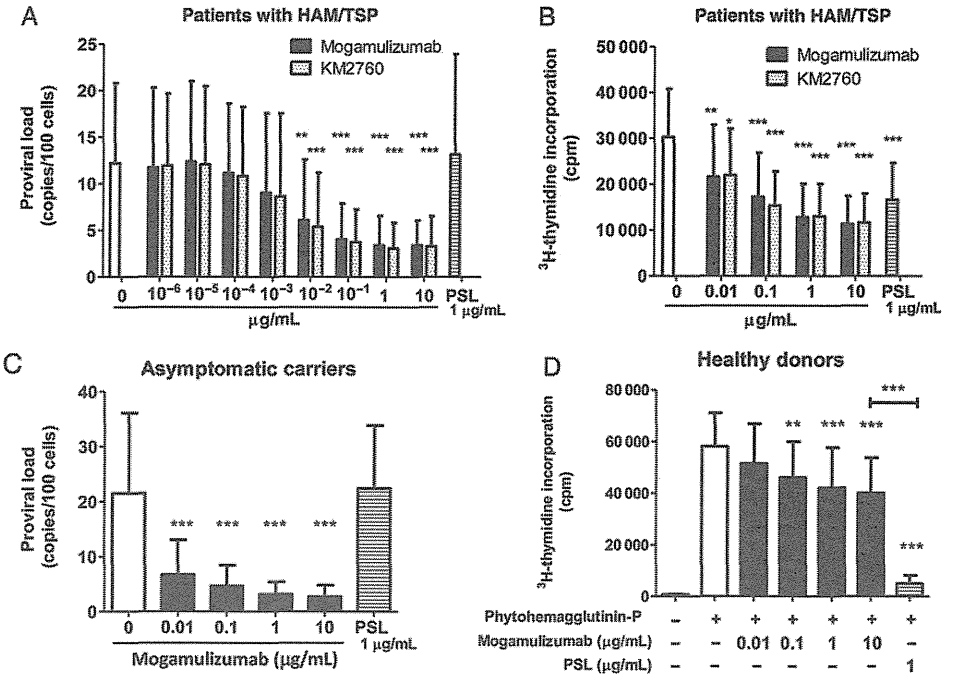

Figure 1. Mogamulizumab and KM2760 reduce the human T-lymphotropic virus type 1 (HTLV-1) proviral load and inhibit spontaneous proliferation of peripheral blood mononuclear cells (PBMCs) from patients with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). *A* and *B*, PBMCs from 11 patients with HAM/TSP were cultured for 7 days without stimuli and without treatment or in the presence of mogamulizumab, KM2760, or prednisolone (PSL). Cells were harvested, and the proviral load was measured using real-time polymerase chain reaction (*A*). ^{3}H-thymidine was added during the last 16 hours of culturing. Cells were then harvested and analyzed for ^{3}H-thymidine incorporation (*B*). Because mogamulizumab and KM2760 were equally effective, only mogamulizumab was used thereafter as representative of the 2. *C*, PBMCs from 8 asymptomatic carriers were cultured for 7 days without treatment or in the presence of mogamulizumab or PSL, and the proviral load was measured as described above. *D*, PBMCs from 8 healthy donors were stimulated with 4 μg/mL of phytohemagglutinin-P and cultured for 3 days without treatment or in the presence of mogamulizumab or PSL. ^{3}H-thymidine incorporation was analyzed as described above. Data are presented as the mean ± SD. Statistical analyses were performed using repeated-measures analysis of variance, followed by the Dunnett test, for comparison with PBMCs alone (*A–C*) or with PBMCs stimulated with PHA (*D*). The paired *t* test was used to compare 10 μg/mL of mogamulizumab and PSL (*D*). **P* < .05, ***P* < .01, and ****P* < .001. Abbreviation: SD, standard deviation.

TSP. In line with previous reports [28], PBMCs produced various cytokines, most notably IFN-γ, in 7-day cultures without stimuli (Figure 2*A*). Mogamulizumab and KM2760 both reduced the production of the proinflammatory cytokines IFN-γ, IL-6, IL-2, and TNF-α, as well as the immunosuppressive cytokine IL-10 (Figure 2*B–F*). Mogamulizumab reduced IFN-γ production more than did human IgG (Supplementary Figure 1*C*). Prednisolone at a concentration of 1 μg/mL effectively reduced IFN-γ and TNF-α but not IL-2, IL-6, or IL-10 production.

Mogamulizumab Eliminates CCR4^{+} Cells Among Both CD4^{+} and CD8^{+} T cells

Mogamulizumab effectively eliminated the CD4^{+}CCR4^{+} T cells in cultured PBMCs from patients with HAM/TSP (Figure 3*A* and 3*B*). FACS analysis also revealed a population of CD4^{-} CCR4^{+} cells similarly affected by the antibody, and these cells were found to be CD8^{+} T cells (Figure 3*C*). Detailed investigation confirmed that CCR4^{+} T cells among the CD8^{+} subset were eliminated by mogamulizumab just as they were from the CD4^{+} subset (Figure 3*D–E*).

The ADCC Activity of Mogamulizumab Is Fast Acting and Specific

FACS analysis showed that mogamulizumab reduced the frequency of CCR4^{+} T cells among both CD4^{+} and CD8^{+} subsets within 6 hours ($P = .0003$ and $P = .004$, respectively), with a similar reduction in Tax^{+} T cells observed within 24 hours ($P = .01$ and $P = .03$, respectively; Figure 4*A–C* and Supplementary Figure 3). By contrast, mogamulizumab did not reduce the frequency of B cells, NK cells, or monocytes after 24 hours (Figure 4*D*).

CD8^{+}CCR4^{+} T Cells From Patients With HAM/TSP Are Numerous and Highly Infected by HTLV-1

CD8^{+}CCR4^{+} T cells were then further analyzed to assess their role in HAM/TSP and predict the potential benefits and risks of eradicating them with mogamulizumab. Samples from patients with HAM/TSP, compared with those from age-matched healthy donors, contained a higher proportion of CCR4^{+} cells among both the CD4^{+} T-cell subset ($P = .003$) and the CD8^{+} T-cell subset ($P = .02$; Figure 5*A*). In addition, the proviral

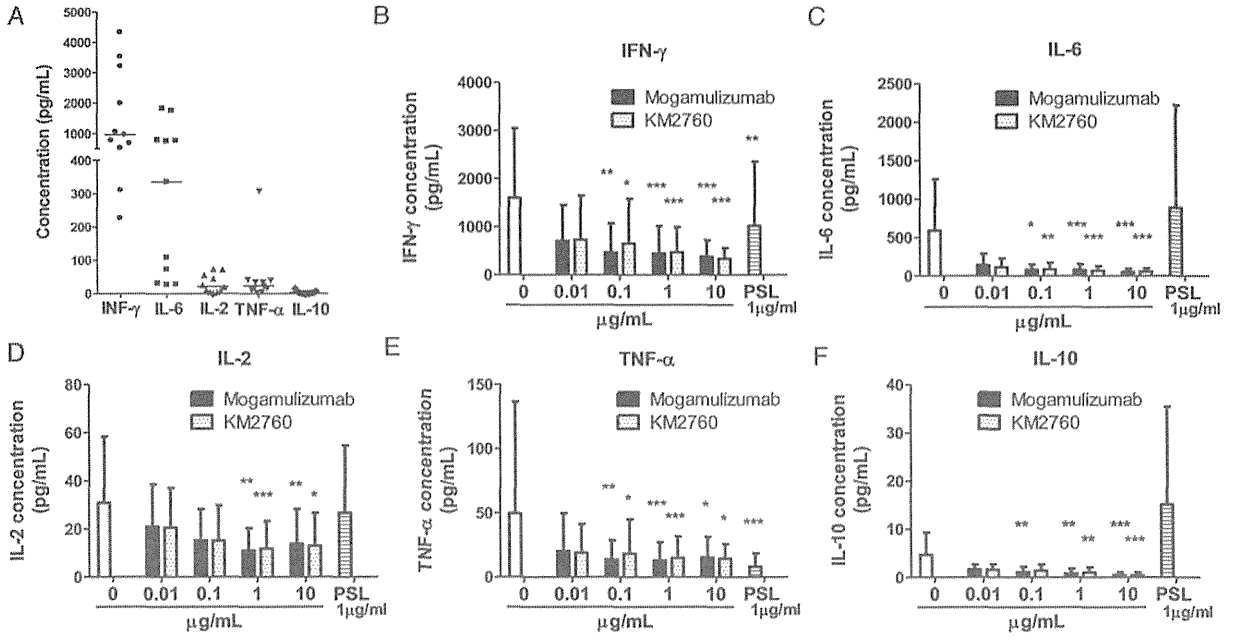

Figure 2. Mogamulizumab and KM2760 inhibit cytokine production in peripheral blood mononuclear cells (PBMCs) from patients with human T-lymphotropic virus type 1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). PBMCs from 11 patients with HAM/TSP were cultured for 7 days without stimuli and without treatment or in the presence of mogamulizumab, KM2760, or prednisolone (PSL). The concentrations of cytokines (interferon γ [IFN-γ], interleukin 6 [IL-6], interleukin 2 [IL-2], tumor necrosis factor α [TNF-α], and interleukin 10 [IL-10]) in the supernatants were then measured. *A*, Direct comparison of the concentrations of these cytokines in the supernatants of untreated PBMC cultures. Horizontal bars represent the median values. *B–F*, The effects of the treatments on the concentrations of these cytokines. Data are presented as the mean ± SD. Statistical analyses were performed using the Friedman test followed by the Dunn test for comparison with PBMCs alone. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Abbreviation: SD, standard deviation.

load was significantly higher in $CD8^+CCR4^+$ T cells than in $CD8^+CCR4^-$ T cells (mean load [±SD], 13.6 ± 7.9 copies/100 cells and 0.72 ± 0.65 copies/100 cells, respectively; $P = .0002$; Figure 5*B*).

$CD8^+CCR4^+$ T Cells From Patients With HAM/TSP Possess Proinflammatory Properties

Here we investigated the functional differences between $CD8^+CCR4^+$ T cells from patients with HAM/TSP and those from healthy donors. $CD8^+CCR4^+$ cells from both groups expressed minimal perforin and granzyme B (Figure 5*C* and 5*D*). In the $CD8^+CCR4^-$ T-cell subset, the frequency of perforin-expressing cells was unremarkable, but the frequency of granzyme B–expressing cells was higher in patients with HAM/TSP than in healthy donors ($P = .04$; Figure 5*C* and 5*D*). Next, cytokine expression was evaluated in PBMCs stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin. Interestingly, samples from patients with HAM/TSP included more IFN-γ–producing cells ($P = .02$; Figure 5*E*) but fewer IL-4–producing cells ($P = .01$; Figure 5*F*) in the $CD8^+CCR4^+$ T-cell subset than did samples from healthy donors. On the other hand, there were no such significant differences among $CD8^+CCR4^-$ T cells (Figure 5*E* and 5*F*).

Finally, the concentrations of cytokines in the supernatants of unstimulated cultures of isolated total $CD8^+$, $CD8^+CCR4^-$, and $CD8^+CCR4^+$ T cells were measured to assess spontaneous cytokine production in these cell populations. Spontaneous IFN-γ production, like spontaneous proliferation, is a hallmark of PBMCs from patients with HAM/TSP [29, 30]. Unsurprisingly, IFN-γ was detected in no cell population from healthy donors. Among samples from patients with HAM/TSP, $CD8^+CCR4^+$ T cells produced remarkably more IFN-γ than did $CD8^+CCR4^-$ cells (mean level [±SD], 364.0 ± 445.3 pg/mL vs 1.9 ± 4.5 pg/mL; $P = .001$; Figure 5*G*). IL-4 was not detected in any of the samples (data not shown).

The Majority of HTLV-1 Tax-Specific Cytotoxic T Lymphocytes Are $CCR4^-$

We analyzed CCR4 expression in Tax-specific CTLs to determine whether CTLs against HTLV-1 also become targets of mogamulizumab. Among the 11 patients studied, 7 had HLA-A*2402 and were analyzed using the HLA-A*2402/HTLV-1 Tax301-309 tetramer. The majority of Tax-specific CTLs did not express CCR4, and the percentage of $CCR4^+$ cells was lower in CTLs than in total $CD8^+$ T cells (mean frequency [±SD], 2.3% ± 1.0% and 8.5 ± 4.7%, respectively; $P = .02$; Figure 6).

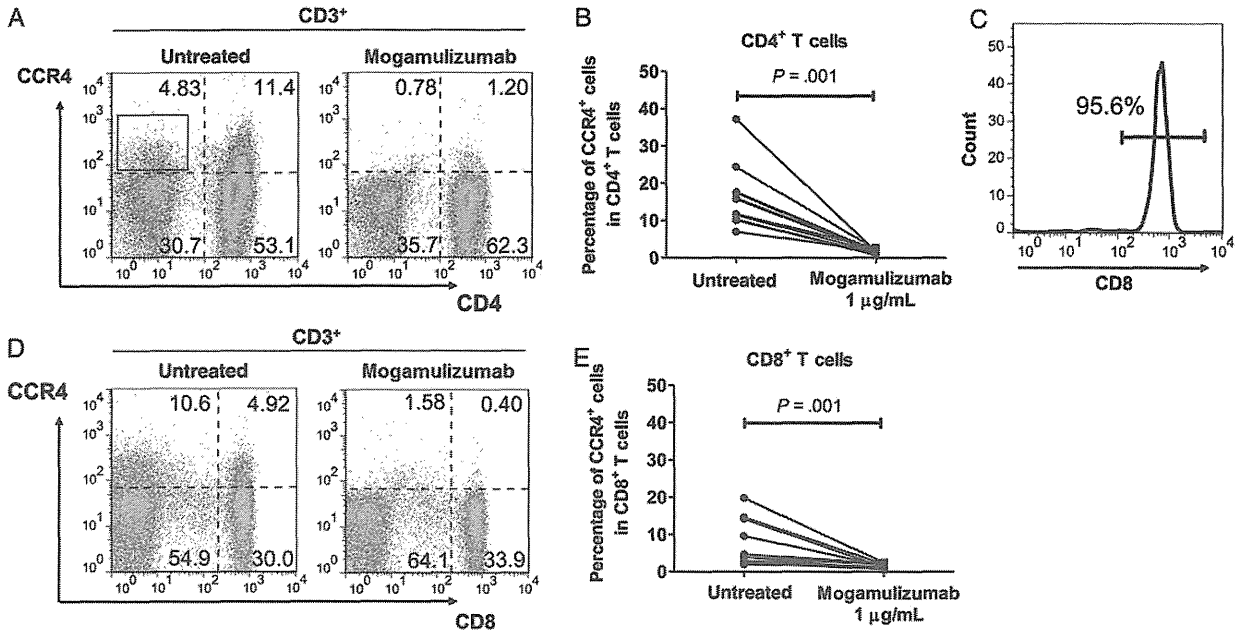

Figure 3. Mogamulizumab eliminates CCR4$^+$ cells in both CD4$^+$ and CD8$^+$ T cells. *A*, Representative dot plots of fluorescence-activated cell-sorter analysis of CCR4 and CD4 expression in CD3$^+$ T cells among peripheral blood mononuclear cells from patients with human T-lymphotropic virus type 1–associated myelopathy/tropical spastic paraparesis after 5-day culture in the presence or absence of 1 μg/mL of mogamulizumab. *B*, Percentages of CCR4$^+$ cells in CD3$^+$CD4$^+$ T cells were compared between the untreated and mogamulizumab groups (n = 11). Statistical analysis was performed using the Wilcoxon signed-rank test. *C*, The population enclosed in the box in panel *A* (the CD3$^+$CD4$^-$CCR4$^+$ subset) was gated and analyzed for the expression of CD8. The percentage of CD8$^+$ cells is shown. *D* and *E*, CCR4 and CD8 expression in CD3$^+$ T cells was analyzed as described above.

DISCUSSION

In this study, we established mogamulizumab as a novel candidate treatment for HAM/TSP that targets infected cells by marking CCR4$^+$ T cells for elimination. Mogamulizumab reduced the number of infected cells, as measured via the proviral load, and thus inhibited the excessive immune responses such as spontaneous proliferation and proinflammatory cytokine production that are attributed to those infected cells (Figures 1 and 2). Effects of mogamulizumab-induced ADCC activity were detectable by FACS after as little as 6 hours of culturing (Figure 4*A*–*C*).

The remaining proviral load after mogamulizumab therapy was higher than expected (mean load [±SD], 3.25 ± 2.58 copies/100 cells; Figure 1*A*). CD4$^+$CCR4$^-$ T cells [18] and CD8$^+$CCR4$^-$ T cells (Figure 5*B*) from patients with HAM/TSP were predominantly uninfected, and the antibody therapy should have destroyed the vast majority of the infected CCR4$^+$ T cells (Figure 3), yielding an expected proviral load of <1.0 copy/100 cells. It is possible that some CCR4$^-$ T cells became infected while the samples were being cultured, which is a potential limitation of such in vitro experiments.

The inhibitory effects of mogamulizumab on PHA-stimulated proliferation in PBMCs from healthy donors were statistically significant but still minimal, compared with those of prednisolone (Figure 1*D*), indicating that mogamulizumab, in contrast to immunosuppressive agents, acts via specific reduction of infected cells rather than via nonspecific immune suppression. Interestingly, prednisolone was considerably less effective at suppressing the proliferation of T cells from patients with HAM/TSP (Figure 1*B*) than from healthy donors. Although we cannot be sure of the reasons behind this discrimination, it appears that spontaneous proliferation is less vulnerable to suppression by steroids since it is not a simple T-cell response to antigens [31, 32]. Nevertheless, compared with mogamulizumab alone, the combination with prednisolone enhanced the suppressive effect of mogamulizumab on spontaneous proliferation without hampering proviral load reduction (Supplementary Figure 2).

Mogamulizumab also reduced the proviral load in PBMCs from asymptomatic carriers (Figure 1*C*), which suggests that it can be administered as a preventive treatment to asymptomatic carriers with high proviral loads who are at risk for developing HAM/TSP or ATL. It is well established that high proviral load is associated with the onset and progression of HAM/TSP [7, 33], as well as with the development of ATL [34].

It was well known that although the main reservoir for HTLV-1 is CD4$^+$ T cells, the virus also infects CD8$^+$ T cells in patients

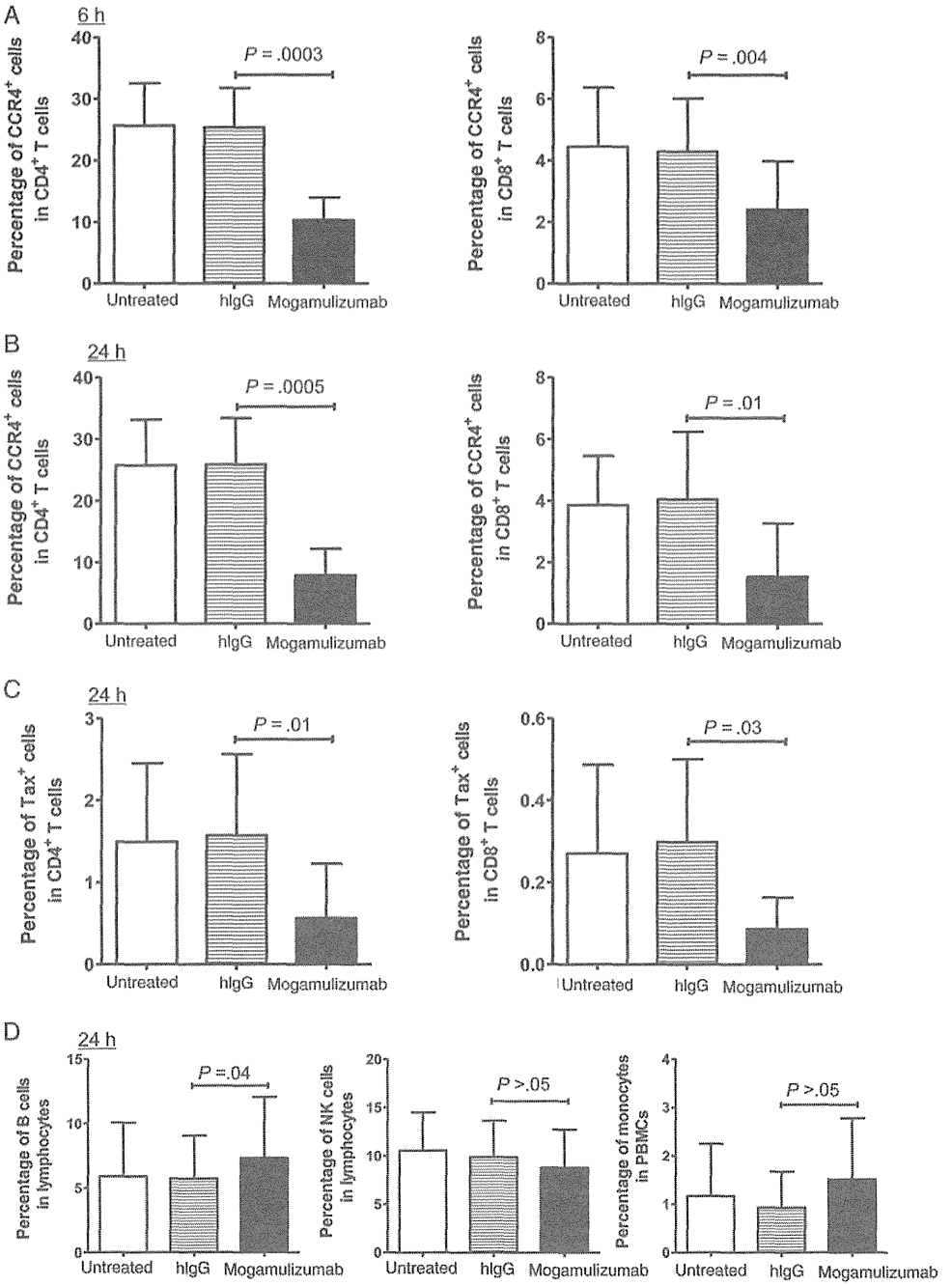

Figure 4. The antibody-dependent cellular cytotoxicity activity of mogamulizumab is fast acting and specific. Peripheral blood mononuclear cells (PBMCs) from 6 patients with human T-lymphotropic virus type–associated myelopathy/tropical spastic paraparesis were cultured in the presence of 1 μg/mL of mogamulizumab or human immunoglobulin G (hIgG) or without treatment. $CD4^+$ and $CD8^+$ T cells were analyzed using fluorescence-activated cell-sorter analysis, and the frequencies of $CCR4^+$ cells after 6 hours (*A*) and 24 hours (*B*), as well as that of Tax^+ cells (*C*) after 24 hours, are shown here. The frequencies of $CD19^+$ B cells and $CD3^-CD56^+$ natural killer (NK) cells among lymphocytes, as well as $CD14^+$ monocytes among PBMCs after 24 hours are also shown (*D*). Data are presented as the mean ± SD. The paired *t* test was used to compare the effects of mogamulizumab and hIgG. Abbreviation: SD, standard deviation.

with HAM/TSP [19]. Here we revealed for the first time that the overwhelming majority of infected $CD8^+$ T cells also expressed CCR4 (Figure 5*B*). Our findings in this study suggest that it is important to eliminate both $CD4^+CCR4^+$ and $CD8^+CCR4^+$ T-cell subsets because both have elevated proviral loads and a tendency to develop proinflammatory traits (Figure 5).

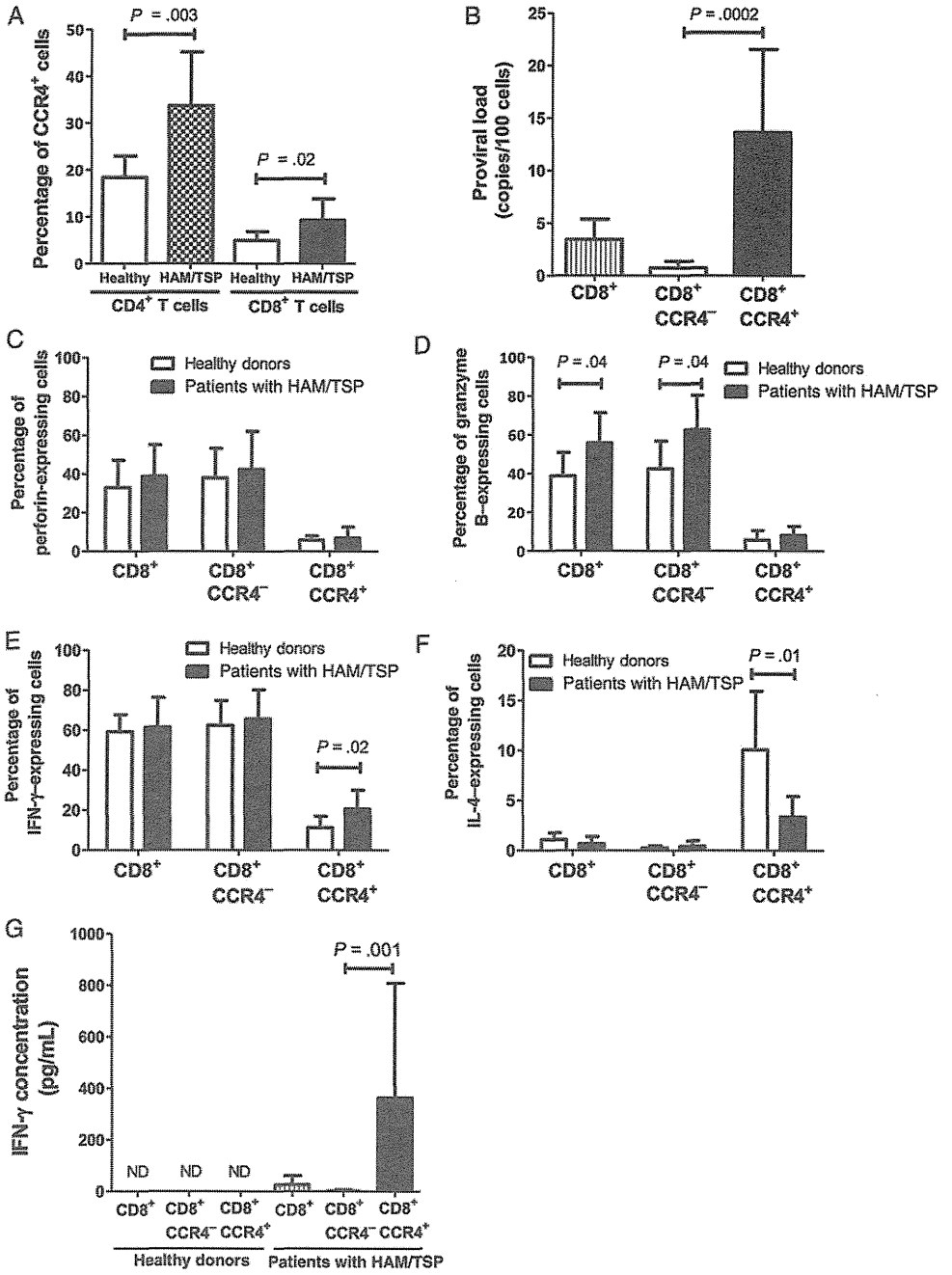

Figure 5. $CD8^+CCR4^+$ T cells from patients with human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) are numerous, highly HTLV-1 infected, and proinflammatory. *A*, Proportions of $CCR4^+$ cells among $CD4^+$ and $CD8^+$ T cells in 8 healthy donors and 11 patients with HAM/TSP were analyzed by fluorescence-activated cell-sorter (FACS) analysis. *B*, The HTLV-1 proviral load in total $CD3^+CD8^+$, $CD3^+CD8^+CCR4^-$, and $CD3^+CD8^+CCR4^+$ T-cell subsets. $CD8^+$ T cells from 11 patients with HAM/TSP were isolated using negative separation with magnetic beads, and then $CD3^+CD8^+$, $CD3^+CD8^+CCR4^-$ and $CD3^+CD8^+CCR4^+$ T cells were separated with FACS analysis. Proviral loads in each subset were measured using real-time polymerase chain reaction. *C–F*, Peripheral blood mononuclear cells (PBMCs) from 8 healthy donors and 11 patients with HAM/TSP were stained for CD8 and CCR4, as well as intracellular perforin or granzyme B, and analyzed using FACS analysis. PBMCs from the same individuals were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL) in the presence of monensin for 6 hours. The cells were then analyzed for CD8, CCR4, and intracellular interferon γ (IFN-γ) or interleukin 4 (IL-4) expressions. The percentages of perforin-expressing (*C*), granzyme B–expressing (*D*), IFN-γ–expressing (*E*), and IL-4–expressing (*F*) cells in total $CD8^+$, $CD8^+CCR4^-$, and $CD8^+CCR4^+$ T-cell subsets from healthy donors versus patients with HAM/TSP are shown. *P* values are indicated only when <.05. *G*, $CD3^+CD8^+$, $CD3^+CD8^+CCR4^-$, and $CD3^+CD8^+CCR4^+$ T cells were isolated from 6 healthy donors and 11 patients with HAM/TSP as described above. These cells (3×10^4 cells/well) were cultured for 3 days without stimuli, and the concentration of IFN-γ in the supernatants was measured. Statistical analysis was performed using the Mann–Whitney *U*-test (*A* and *C–F*) or the Wilcoxon signed-rank test (*B* and *G*). Data are presented as the mean ± SD. Abbreviation: SD, standard deviation.

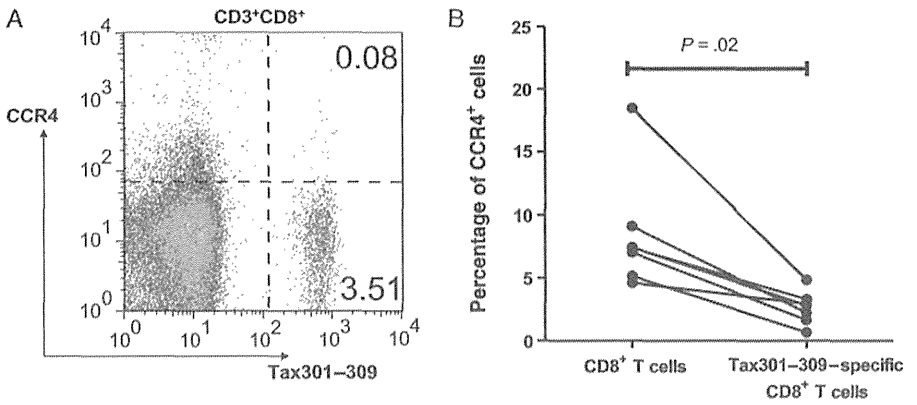

Figure 6. $CD8^+CCR4^+$ T cells from patients with human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) do not include many Tax-specific cytotoxic T lymphocytes (CTLs). *A*, A representative dot plot of fluorescence-activated cell-sorter (FACS) analysis of the expression of CCR4 in HTLV-1 Tax-specific CTLs. Peripheral blood mononuclear cells from a patient with HAM/TSP and HLA-A*2402 were stained with antibodies for CD3, CD8, CCR4, and HLA-A*2402-restricted Tax301-309–specific tetramer. The $CD3^+CD8^+$ subset was gated. The values in the upper and lower right quadrants indicate the percentages of $CCR4^+$ and $CCR4^-$ Tax-specific CTLs among $CD3^+CD8^+$ T cells, respectively. *B*, Proportions of $CCR4^+$ cells among total $CD8^+$ T cells and Tax301-309–specific $CD8^+$ T cells are compared (n = 7). Statistical analysis was performed using the Wilcoxon signed-rank test.

$CD8^+CCR4^+$ T cells normally produce IL-4 more often than IFN-γ and hardly produce any cytotoxic granules [35, 36]; these cells are thought to be protective against type 1-skewed inflammation [21, 37]. In patients with HAM/TSP, these $CD8^+CCR4^+$ but not $CD8^+CCR4^-$ T cells are altered to produce IFN-γ rather than IL-4 (Figure 5*E* and 5*F*). $CD8^+CCR4^+$ T cells cultured alone exhibited spontaneous IFN-γ production (Figure 5*G*), a hallmark of PBMCs from patients with HAM/TSP [29, 30]. These results suggest that abnormal cells contributing to the pathogenesis of HAM/TSP exist not only among $CD4^+CCR4^+$ T cells but also among $CD8^+CCR4^+$ T cells. It is thought that the functional abnormalities of these cells may arise through transformations occurring within the infected cells themselves, whereby HTLV-1 Tax induces transcriptional alterations via T box transcription factor [18].

In the present study, HTLV-1 infection did not influence cytotoxic granule production in $CD8^+CCR4^+$ T cells (Figure 5*C* and 5*D*). The slightly increased fraction of granzyme B^+ cells in $CD8^+CCR4^-$ T cells from patients with HAM/TSP is presumably attributable to the immune activation resulting from the chronic viral infection [38–40].

Although eliminating the abnormal immune responses of the infected cells should alleviate inflammation and related symptoms of the infection, it is also true that immune responses against HTLV-1 are important for controlling said infection [41]. We evaluated CCR4 expression in HTLV-1 Tax-specific CTLs for fear that use of mogamulizumab might inadvertently destroy CTLs that would have helped to control the infection [42, 43]. Since Tax-specific CTLs have been reported to be preferentially infected by HTLV-1 [44], there was some concern that our finding that infected $CD8^+$ T cells are predominantly $CCR4^+$ meant that these CTLs would also be targeted by mogamulizumab. However, we found that the majority of Tax-specific CTLs do not express CCR4 (Figure 6), meaning that they should essentially be spared during mogamulizumab treatment.

Also concerning is that mogamulizumab is expected to target $CD4^+CCR4^+$ regulatory T (Treg) cells [45], which could elicit autoimmune problems and even exacerbate the chronic inflammation plaguing patients with HAM/TSP. However, there are also $CCR4^-$ Treg cells [45], which would be spared, and there have been no reports of increased incidence of autoimmune disease in patients with ATL treated with mogamulizumab. Furthermore, reducing the number of Treg cells may benefit patients with HAM/TSP by preventing abundant Treg cells from dampening immune control of the HTLV-1 infection [28, 46].

We expect that eliminating HTLV-1–infected cells in the peripheral blood with mogamulizumab would reduce the number of proinflammatory cells and mitigate the inflammation in the CNS. Although HAM/TSP is a disease of the CNS, recent reports suggest that it is indeed effective to target HTLV-1–infected cells in the peripheral blood because continued migration of infected cells from the peripheral blood maintains and even exacerbates the inflammation in the CNS [30].

Based on the results of this study, we have begun conducting a clinical trial to test the efficacy of mogamulizumab on patients with HAM/TSP (UMIN000012655). Our data suggest that as little as one thousandth of the dose administered to patients with ATL (1 mg/kg body weight [13]) may be effective for patients with HAM/TSP. In contrast to patients with an aggressive cancer such as ATL, those with a chronic inflammatory disorder like HAM/TSP would benefit from a more conservative approach that is safer but still effective.