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
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RESEARCH ARTICLE

Regulatory T Cell Responses to High-Dose Methylprednisolone in Active Systemic Lupus Erythematosus

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Abstract

Background/Purpose

A slight increase in the proportion of circulating regulatory T (Treg) cells has been reported in systemic lupus erythematosus (SLE) patients taking oral prednisone. The effects of intravenous (IV) high dose methylprednisolone (MP) on Tregs have not yet been described, especially in active SLE.

Methods

We prospectively analyzed the proportion of circulating CD4⁺ Treg cell subsets defined as follows: (1) naïve Treg (nTreg) FoxP3^{low}CD45RA⁺ cells; (2) effector Treg (eTreg) FoxP3^{high}CD45RA⁺ cells; and (3) non-suppressive FoxP3^{low}CD45RA⁺ cells (non-regulatory Foxp3^{low} T cells). Peripheral blood mononuclear cells of patients with active SLE were analyzed before the first infusion of IV high dose MP (day 0) and the following days (day 1, day 2, ±day 3 and ±day 8). The activity of SLE was assessed by the SLEDAI score.

Results

Seventeen patients were included. Following MP infusions, the median (range) percentage of eTregs significantly increased from 1.62% (0.53–8.43) at day 0 to 2.80% (0.83–14.60) at day 1 ($p = 0.003$ versus day 0), 4.64% (0.50–12.40) at day 2 ($p = 0.06$ versus day 1) and 7.50% (1.02–20.70) at day 3 ($p = 0.008$ versus day 2), and declined to baseline values at day 8. Expanding eTreg cells were actively proliferating, as they expressed Ki-67. The frequency of non-regulatory FoxP3^{low} T cells decreased from 6.39% (3.20–17.70) at day 0 to 4.74% (1.03–9.72) at day 2 ($p = 0.005$); nTreg frequency did not change. All patients

Competing Interests: The authors have declared that no competing interests exist.

clinically improved immediately after MP pulses. The absence of flare after one year of follow up was associated with a higher frequency of eTregs at day 2.

Conclusion

IV high dose MP induces a rapid, dramatic and transient increase in circulating regulatory T cells. This increase may participate in the preventive effect of MP on subsequent flares in SLE.

Introduction

FoxP3-expressing regulatory T (Treg) cells are instrumental for the maintenance of self-tolerance. While the absence of Treg cells in scurfy mice and IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked) patients bearing a dysfunctional FOXP3 gene leads to severe multisystemic lethal autoimmune disease [1–3], transfer of T cells devoid of Treg cells in nude mice leads to milder systemic autoimmunity, including gastritis, oophoritis and sometimes clinical and biological features resembling systemic lupus erythematosus (SLE), including arthritis, nephritis and the production of anti-double stranded DNA [4–6].

The seminal finding that a lack of Treg cells in adult mice could provoke a SLE-like disease in mice has led to numerous studies focused on Treg cell modifications in SLE.

Treg cells were first defined in humans as CD4⁺T cells harboring the alpha chain of the IL-2 receptor i.e., CD25 [7], following the seminal description by Sakaguchi *et al.* of Treg cells in mice [6]. The studies that have followed demonstrated that such Treg cells in humans also expressed the FoxP3 transcription factor [7]. However, subsequent studies have demonstrated that human FoxP3 expressing cells are heterogeneous in phenotype and function *i.e.*, some circulating FoxP3 expressing cells are not suppressive [8, 9]. FoxP3⁺CD4⁺ regulatory T cells can be divided into CD4⁺CD45RA[−]FoxP3^{bright}CD25^{bright} and CD4⁺CD45RA⁺FoxP3⁺CD25⁺ fractions, while the remaining CD4⁺FoxP3⁺ (i.e., CD4⁺CD45RA[−]FoxP3^{low}CD25⁺) include a notable amount of non-regulatory, cytokine-secreting, activated T cells (= *non-regulatory FoxP3⁺ T cells*). CD4⁺CD45RA[−]FoxP3^{bright}CD25^{bright} Tregs are activated, highly suppressive and *in vivo* proliferating cells defined as *effector Tregs* (eTregs [8]) while CD4⁺CD45RA⁺FoxP3⁺CD25⁺ Tregs are fully functional and referred to as *naïve Tregs* (nTregs [8]). We have shown that the latter were highly increased during SLE flares, while effector Treg cells were decreased in most patients with SLE flares [8, 10]. These results are in line with numerous published reports showing an imbalance between Treg cells and effector T cells in active SLE [11, 12]. Numerous studies have also shown that the number of Treg cells returns to normal values when the disease is inactive [5, 10, 13]. Therefore, the manipulation of Treg cells to increase their number is considered an interesting potential therapeutic strategy to develop in SLE.

Administration of glucocorticoids is commonly used and has been proven efficient as a treatment for SLE flares irrespective of organ involvement [14, 15]. In severe flares, intravenous (IV) high dose methylprednisolone (MP) is useful to induce a rapid suppression of acute inflammation [16–19]. Hence, IV high dose MP pulses are recommended as part of the initial treatment regimen of severe lupus nephritis [20, 21] and can also be useful to obtain rapid beneficial effects on several types of non-renal lupus erythematosus [16–19]. While the broad actions of glucocorticoids on lymphocytes, neutrophils, mononuclear phagocytes and cytokines to induce anti-inflammatory and immunosuppressive effect are well known [19, 22, 23], their impact on Treg cells is less documented. Several studies have suggested that the induction

of Treg cells may contribute to the immunosuppressive effects of glucocorticoids [24–28]. In SLE, a slight increase in the proportion of circulating Treg cells has been reported in patients taking oral prednisone [29–31]; the time to Treg cell recovery was reduced in patients treated with IV high dose MP [13]. However, to our knowledge, there has been no detailed report on the short-term effects of IV high dose MP on the different subsets of FoxP3⁺ T cells in active SLE until now.

Here, we show that IV high dose MP leads to a rapid, marked and transient increase in circulating effector Treg cells in most patients with active SLE. We also show that the expansion in effector Treg cells is associated with a better clinical outcome after one-year follow-up i.e., the absence of subsequent flares.

Methods

Patients

We conducted a prospective observational study between September 2011 and May 2013 in the department of internal medicine 2 (French national center for lupus and antiphospholipid syndrome) at Pitié Salpêtrière hospital, Paris, France. The inclusion criteria were to fulfill at least four of the 1997 American College of Rheumatology criteria for SLE [32] and to be treated for an SLE flare with high dose IV MP infusion (500 or 1000 mg repeated daily for 3 consecutive days, i.e., day 0, day 1 and day 2). At baseline, the clinical data and the Safety of Estrogens in Lupus Erythematosus National Assessment—Systemic Lupus Erythematosus Disease Activity Index (SELENA-SLEDAI) [33–35] were recorded. Routine measures were used to determine the anti-dsDNA antibodies titer (by Farr assay), anti-SSA/Ro, anti-SSB/La, anti-Sm, anti-RNP, anti-ribosome and anti-cardiolipin autoantibodies, the complement C3 level, complete blood count, urinary casts, proteinuria, pyuria and hematuria. Whenever required by the specific clinical situation, a complete blood count was also performed at day 2 which allowed us to determine the absolute count of lymphocytes at this date (pts # 1, 3, 4, 6, 8, 9, 11, 13, 16 and 17). After discharge from the hospital, all patients were closely monitored, initially on a monthly basis, for one year by a physician of the department of internal medicine 2. Clinical and biological data were systematically collected at 3, 6 and 12 months (month 3, month 6 and month 12) after MP pulses. Patients were asked to see their physician if a lupus flare occurred between the consultations. Lupus flares were defined according to the SELENA-SLEDAI Flare Index (SFI) [34, 35]. Bad responders were defined as patients having at least one lupus flare (mild, moderate or severe) during the one year follow-up after the high dose MP. The study was ethically approved by the CPP (Comités de Protection des Personnes) Ile de France VI and has been conducted according to the principles expressed in the Declaration of Helsinki. Patients provided written informed consent prior to their participation.

Cell isolation and Flow Cytometry

Blood samples were collected into ACD (citric acid, citrate, dextrose) tubes before the first pulse of MP (baseline or day 0) and the following days until the patient's discharge from the hospital. We were unable to collect blood samples at day 3 and day 8 for the patients discharged from the hospital before this time point. Whole peripheral blood mononuclear cells (PBMCs) were isolated through a Ficoll gradient (Eurobio, Les Ullis, France) and analyzed by flow cytometry (FACS Canto II, BD Bioscience). PBMCs were surface-stained with monoclonal antibodies: PerCP-conjugated-anti-CD4, APC-H7-conjugated-anti-CD45RA, BV450-conjugated-anti CD127, PeCy7-conjugated-anti CD25 (all from BD bioscience). Cells were then fixed and permeabilized using a fix/perm buffer (eBioSciences) following the manufacturer's instructions and then intracellularly stained with PE-conjugated-anti-FoxP3 (259D clone) and

FITC-conjugated-anti-Ki67 (BD Bioscience). The FoxP3 expressing CD4⁺ subset phenotype was defined as previously shown [8]. Naïve Treg cells were defined as CD4⁺CD45RA⁺FoxP3^{low} cells (nTreg) and effector Treg cells were defined as CD4⁺CD45RA⁺FoxP3^{high} cells (eTreg), while FoxP3 expressing non-regulatory Treg CD4⁺ T cells were defined as CD4⁺CD45RA⁺FoxP3^{low} cells (non-reg FoxP3⁺ T cells).

Statistical analysis

Values for quantitative variables were expressed as the median and range. Differences between groups were tested using the nonparametric Mann-Whitney U test and the Wilcoxon matched pairs signed ranks test. Statistical analyses were performed using GraphPad Prism, version 5.02 software (GraphPad Software, San Diego, CA, USA). All tests were 2-sided and a *p* value < 0.05 was considered statistically significant.

Results

Patient characteristics

Seventeen patients (pts) were included. Their baseline characteristics are described in Tables 1 and 2. All pts with a previously established SLE diagnosis were taking hydroxychloroquine (HCQ) except one. Eleven patients were treated with prednisone including nine with a dose

Table 1. Baseline characteristics and disease parameters of the SLE patients*.

	Patientsn = 17
Women	16 (94.1)
Age, median (range) years	34 (18–61)
Disease duration, median (range) years	5 (0–15)
<i>Previous organ involvement</i>	
Mucocutaneous or Musculoskeletal	12 (70.6)
Renal	2 (11.8)
Cardiorespiratory	2 (11.8)
Neuropsychiatric	2 (11.8)
Hematological	1 (5.9)
Daily prednisone	11 (64.7)
Hydroxychloroquine	13 (76.4)
Immunosuppressive agents [†]	5 (29.4)
No treatment	3 (17.7)
Positive Farr assay	13 (76.4)
Anti-SSa positive	8 (47.1)
Anti-SSb positive	3 (17.7)
Anti-Sm positive	6 (35.3)
Anti-RNP positive	7 (41.2)
Anti-ribosome positive	3 (17.7)
Anti-cardiolipin positive	8 (47.1)
Low C3	10 (58.8)
SELENA-SLEDAI score, median (range)	10 (2–26)

* except where indicated otherwise, values are the number (%) of patients. SLE = Systemic Lupus Erythematosus; SELENA-SLEDAI = Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of the SLE Disease Activity Index (SLEDAI).

[†] Excluding antimalarial and prednisone.

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Table 2. Baseline characteristics, disease parameters and treatment of the SLE patients.

#	Sex	Age (yrs)	Baseline characteristics (day 0)					Dose of methylprednisolone [†] (mg)	Immediate treatment after the high dose of methylprednisolone		Treatment at month 12	
			Disease duration (yrs)	Daily Pred (mg)	Other treatments	Organ involvement	SLEDAI score*		Daily Pred (mg)	Other treatments	Daily Pred (mg)	Other treatments
1	F	56	4	10	MTX	Rash, arthritis	6	500	10	MTX, BMB	10	MTX
2	F	25	9	0	HCQ	GN (class IV), arthritis	24	1000	65	HCQ, MMF	10	HCQ, MMF
3	M	18	0	0	0	GN (class V), fever, rash, pleurisy, encephalitis	26	1000	55	HCQ, CYC	5	HCQ, MMF
4	F	29	7	5	HCQ	GN (class II), arthritis	14	1000	15	HCQ	5	HCQ
5	F	61	5	5	HCQ	Arthritis, myelitis	4	1000	65	HCQ, AZA	5	HCQ, MMF
6	F	54	8	5	HCQ, MMF	GN (class III), alopecia	14	1000	50	HCQ, CYC	20	HCQ
7	F	37	9	0	HCQ	Alopecia, arthritis	10	500	20	HCQ	10	HCQ
8	F	34	1	10	HCQ	GN (class III)	16	1000	30	HCQ, MMF	5	HCQ, MMF
9	F	33	0	0	0	Arthritis, aseptic, meningitis, intracranial hypertension	8	1000	60	HCQ	10	HCQ
10	F	23	1	30	HCQ	GN (class V), arthritis, alopecia	14	1000	30	HCQ	15	HCQ, MMF
11	F	30	2	20	HCQ, MMF	fever, arthritis, pericarditis	9	1000	20	HCQ	10	HCQ
12	F	38	6	9	HCQ, MTX	Arthritis	6	1000	20	HCQ, MTX	15	HCQ, MTX
13	F	52	2	5	HCQ, MTX	GN (class III), arthritis, alopecia, pericarditis	20	1000	70	HCQ, CYC	5	HCQ
14	F	39	13	5	HCQ	Pleurisy, arthritis	10	1000	20	HCQ	5	HCQ
15	F	42	0	0	0	Myelitis	2	1000	40	HCQ, CYC	5	HCQ, AZA
16	F	31	9	10	HCQ	mucosal ulcers, arthritis	8	500	15	HCQ, AZA	10	HCQ, MTX
17	F	28	15	0	HCQ	sudden deafness	10	1000	50	HCQ	6	HCQ

Pred = prednisone; MTX = methotrexate; BMB = belimumab; HCQ = hydroxychloroquine; MMF = mycophenolate mofetil; CYC = cyclophosphamide; AZA = azathioprine; GN = glomerulonephritis

* using the SELENA-SLEDAI [Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of the SLE Disease Activity Index (SLEDAI)].

[†] the dose was delivered intravenously daily for three consecutive days.

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of ≤ 10 mg/day. Immunosuppressive treatment was as follows: mycophenolate mofetil for 2 pts and methotrexate for 3 pts. All pts required hospitalization for SLE activity and were therefore considered to have a severe flare in the SELENA-SLEDAI Flare composite score [34, 35]. At inclusion, 12 pts had active arthritis and 7 had renal involvement (class III 3 pts, class V 2 pts, class IV 1 pt and class II 1 pt). Five pts developed a neuro-psychiatric involvement for which other causes than lupus were excluded (myelitis 2 pts, intracranial hypertension 1 pt,

encephalitis 1 pt, sudden deafness 1 pt). Four pts had a pleurisy or a pericarditis. Patient #9 suffered from intracranial hypertension (nausea, vomiting, headache and blurred vision due to severe bilateral papilledema with raised intracranial pressure, aseptic meningitis and a normal MRI scan without cerebral venous sinus thrombosis), arthritis, positive Farr assay, positive Sm and low complement. The four pts (pts #1, 7, 12 and 16) for whom the musculoskeletal involvement was the main manifestation of the lupus flare suffered from a worse or new severe arthritis with an active synovitis ≥ 12 joints with marked loss of functional range of movements and significant impairment of activities of daily living, that has been present on several days. They all had to stop their professional activity due to the ongoing arthritis. Patients # 1, 7 and 16 received a lower dose of MP infusion, i.e., 500 mg per day for 3 consecutive days, while the fourteen other pts were treated with 1000 mg per day for 3 consecutive days. After the MP infusion, the baseline dose of prednisone was increased in 14 pts and a new immunosuppressant was introduced in 9 pts: cyclophosphamide for 4 pts, mycophenolate mofetil for 2 pts, azathioprine for 2 and belimumab for 1 patient (Table 2).

Effector Treg expansion following IV methylprednisolone pulses

At baseline, in accordance with our published works [8, 10], the median (range) percentages of CD45RA⁺FoxP3^{high} eTreg, CD45RA⁺FoxP3^{low} nTreg and non-regulatory FoxP3^{low} T cells were 1.62% (0.53–8.43), 2.48% (0.87–5.58) and 6.39% (3.20–17.70), respectively (Table 3 and Fig 1).

Following MP therapy, we observed a marked increase in the eTregs subset in all patients except pt #10 (Table 3 and Figs 1 and 2A). One representative patient FoxP3⁺CD4⁺ T cells subset kinetics is shown (Fig 1). The median percentage of CD45RA⁺FoxP3^{high} eTregs significantly increased at day 1 at 2.80% (0.83–14.60) ($p = 0.003$ versus day 0) (data not shown) and even more at day 2 [4.64% (0.50–12.40), $p = 0.0005$ versus day 0 and $p = 0.06$ versus day 1] as well as at day 3 [7.50% (1.02–20.70), $p = 0.008$ versus day 0 and $p = 0.008$ versus day 2] (Table 3, Fig 2A and data not shown). The increase in the proportion of eTregs among CD4⁺ T cells that we observed at day 2 corresponds to a significant increase in the count of eTreg cells (S1 Fig). The range of the expansion was variable. While most patients doubled their proportion of eTreg cells from baseline values at day 2 (Table 3), we observed that some patients displayed an increase in eTreg cells superior to a 5-fold at either D2 or D3 (e.g., patient #4 at day 2 and patients #2, 3 and 6 at day 3) and other patients showed an increase below 1.6 (pts #7, 9, 11, 12, 13, 15 and 16). Thus, these results indicate that high dose IV MP induces a variable but significant increase in circulating eTreg cells.

When analyzing the phenotype of eTreg cells at day 2 and day 3, we observed that most eTreg cells expressed the intranuclear proliferation marker Ki-67, indicating that MP may ultimately result in increased eTreg cell proliferation (Fig 1). Approximately 90% of eTregs cells at day 2 expressed CD15s, a marker that is highly specific for activated, terminally differentiated, and suppressive eTreg cells (S2 Fig) [36]. In pts treated with MP, only a small proportion of eTregs expressed CD45RA (Fig 1) and only a very small subset of CD45RA⁺ CD4⁺ T cells were Ki-67⁺ (data not shown). Effector Tregs did not express Annexin V (data not shown). Because we also observed a small decrease in nTreg cells, although not statistically significant (Fig 2B), we cannot exclude a concomitant enhanced conversion of nTreg cells to eTreg cells.

Finally, the expansion of eTregs was transient, as their proportion declined to baseline values by day 8 following the first pulse of MP in all of the 6 patients who were evaluated (Fig 2A). For these 6 patients, the median frequencies of eTreg cells increased from 1.56% (0.97–5.80) at day 0 to 6.92% (4.23–9.55) at day 3 (for the two patients for whom the day 3 data are missing, the eTreg cells frequencies at day 2 were taken instead) ($p = 0.031$ versus day 0) only to

Table 3. Evolution of FoxP3⁺ T cell subsets and disease activity in SLE patients following high dose methylprednisolone.

#	effector Tregs				naïve Tregs			non-reg FoxP3 ⁺ T cells			SLEDAI score*			No. of flares at month 12
	day 0 [†]	day 2	day 3	fold increase [‡]	day 0	day 2	day 3	day 0	day 2	day 3	month 3	month 6	month 12	
1	1.43	6.22		4.35	2.76	0.63		5.22	3.90		0	0	0	1 mild or moderate (Rash at M7)
2	1.33	3.63	9.55	2.73	1.78	0.80	1.64	6.39	4.14	3.15	6	2	2	0
3	1.62	4.64	8.09	2.86	2.29	3.30	3.35	10.72	4.74	5.53	4	4	2	0
4	1.35	6.83		5.06	3.99	4.26		6.61	6.94		2	2	2	0
5	0.97	3.49	4.23	3.60	1.41	5.77	3.33	3.69	2.91	2.71	0	0	0	1 severe (Myelitis at M3)
6	1.64	7.97	9.24	4.86	5.58	1.85	0.64	7.70	4.83	3.65	10	10	6	0
7	8.43	12.40	20.70	1.47	0.94	0.83	0.74	16.50	6.97	9.00	4	4	4	0
8	0.53	1.07		2.02	2.86	3.73		3.20	2.62		12	6	2	0
9	5.80	6.04	6.90	1.04	4.50	2.73	1.90	17.70	5.40	5.40	2	2	2	0
10	0.92	0.50	1.02	0.54	3.32	1.65	1.63	6.34	1.03	1.85	4	6	0	3 severe (Arthritis at M1, M6 and M9)
11	1.90	2.66		1.40	3.87	0.53		8.07	4.03		2	2	2	1 mild or moderate (Arthritis at M11)
12	1.31	2.10		1.60	0.87	0.43		4.84	3.76		6	6	6	5 mild or moderate (Arthritis at M3, M6, M8, M9 and M10)
13	2.59	3.64	3.84	1.41	2.71	6.78	2.77	10.58	9.10	8.46	10	2	2	0
14	1.64	6.30		3.84	0.93	0.69		14.07	6.60		4	4	4	0
15	4.25	6.74		1.59	2.45	2.40		5.70	9.72		0	0	0	0
16	1.84	2.87		1.56	2.48	2.90		5.14	6.13		4	8	8	1 severe (arthritis at M6) 1 mild to moderate (arthritis at M12)
17	1.49	6.94		4.66	1.03	0.50		6.39	4.66		2	2	2	0

* using the SELENA-SLEDAI [Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of the SLE Disease Activity Index (SLEDAI)].

[†] values are the percentage of the subset among CD4⁺ T lymphocytes.

[‡] values are the ratio of effector Tregs between day 2 and day 0.

[¶] flares occurring during the 12 months which followed the high dose methylprednisolone were defined according to the SELENA-SLEDAI Flair Index (SFI).

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decrease to 1.43% (0.90–2.30) at day 8 ($p = 0.031$ versus day 2/3 and $p = 0.60$ versus day 0) (Fig 2A).

Non-regulatory FoxP3⁺ T cells markedly decrease following IV methylprednisolone pulses

A significant decrease in the median frequency of non-regulatory CD45RA⁺FoxP3^{low} T cells was observed from 6.39% (3.20–17.70) at day 0 to 4.74% (1.03–9.72) at day 2, $p = 0.005$, and 4.53% (1.85–9.00) at day 3, $p = 0.008$ (Table 3 and Fig 2C). Non-regulatory FoxP3 T cells did not express Annexin V (data not shown).

Disease improvement after methylprednisolone pulse

Following the IV MP infusions and the subsequent changes in the daily treatment, all patients initially improved (Table 3, Fig 3A and data not shown). At month 3, the SLEDAI score improved compared to baseline for all pts except patient #12. The median SLEDAI score

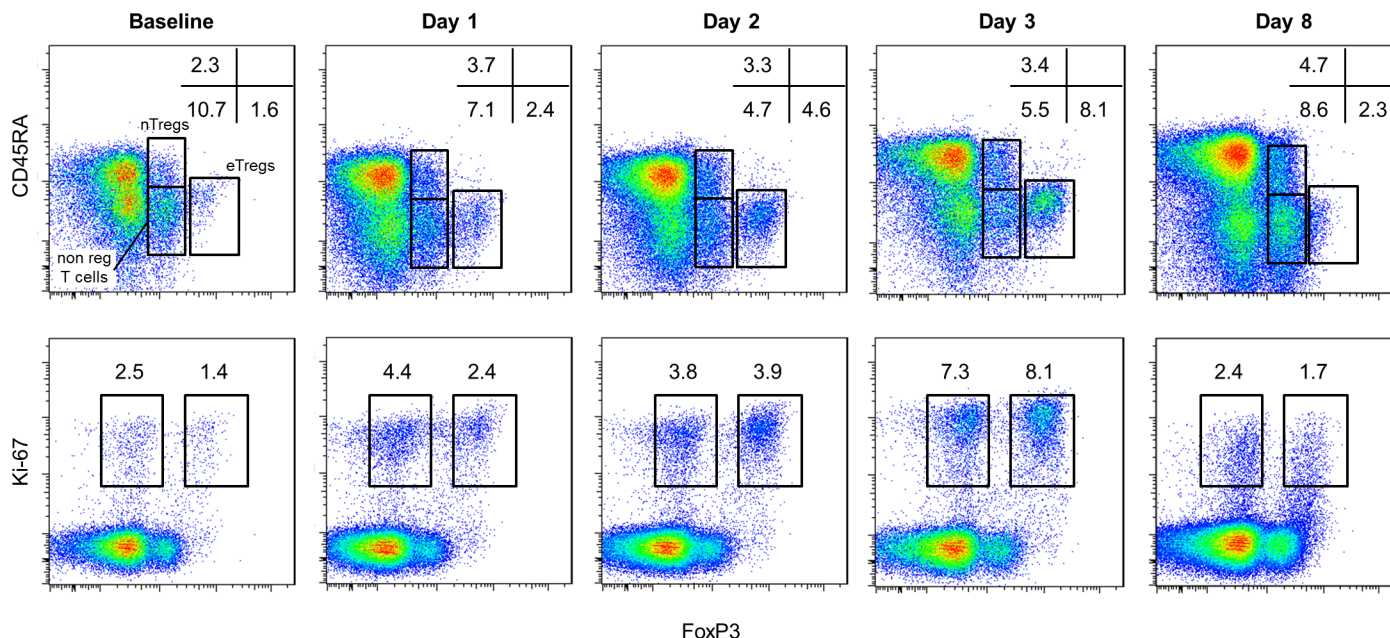


Fig 1. Cytofluorometric analysis of CD4⁺FoxP3⁺ T cell subsets following IV methylprednisolone pulses in SLE patients. Fresh PBMCs from SLE patients were analyzed by flow cytometry, gated on CD4⁺ T lymphocytes, for the expression of FoxP3 and CD45RA (top rows) and Ki-67 (bottom rows). FoxP3⁺CD4⁺ T cells can be divided into CD4⁺CD45RA⁺FoxP3^{high} effector Tregs (eTregs) and CD4⁺CD45RA⁺FoxP3^{low} naive Tregs (nTregs), while the remaining CD4⁺CD45RA⁺FoxP3^{low} include a notable amount of non-regulatory, cytokine-secreting, activated T cells (non-regulatory FoxP3⁺ T cells) [8]. FoxP3^{high}Ki-67⁺ (right gate), which correspond to the eTregs, and FoxP3⁺Ki-67⁺ (left gate), which correspond to non-regulatory T-cells, are shown on the lower FACS panel. Percentages of the different subsets are shown. Representative analyses from one SLE patient are shown (pt #3).

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decrease from 10 (2–26) at day 0 to 4 (0–12) at month 3, $p = 0.0005$. However, this beneficial effect was not maintained for all patients: 3 pts experienced an early relapse (pts #5, 10 and 12) and, after one year of follow-up, 3 more pts had experienced at least one new lupus flare (pts #1, 11 and 16) (Table 3). Thus, six patients were classified as bad responders one year after MP pulse therapy. The evolution of the SLEDAI score according to the responder status is displayed in Fig 3. Even the patients classified as bad responders showed a slight, but not significant, improvement in disease activity (Fig 3A).

eTreg expansion is associated with a better clinical outcome

Several lines of evidence have shown that regulatory T cells can prevent the occurrence of autoimmune responses. As a result, the use of Treg cell expansion has become an attractive target for the prevention of autoimmune disorders [37]. We therefore investigated whether the expansion of eTreg cells following IV MP pulses was associated with the absence of subsequent flares. The proportions of eTreg cells among CD4⁺ T cells two days after IV MP pulses were higher in the patients with a good clinical response during the year which followed the MP pulses (Fig 3B). We also compared the fold expansion of eTreg cells at day 2 between patients with good responses and those with a poor outcome at month 12 and found no significant difference (Fig 3B).

Discussion

It is known that glucocorticoids, commonly used to treat SLE, exert a broad range of effects on the immune system. As some studies have shown that oral steroid drugs could also influence the regulatory cell population, we postulated that IV high dose MP could have a dramatic effect

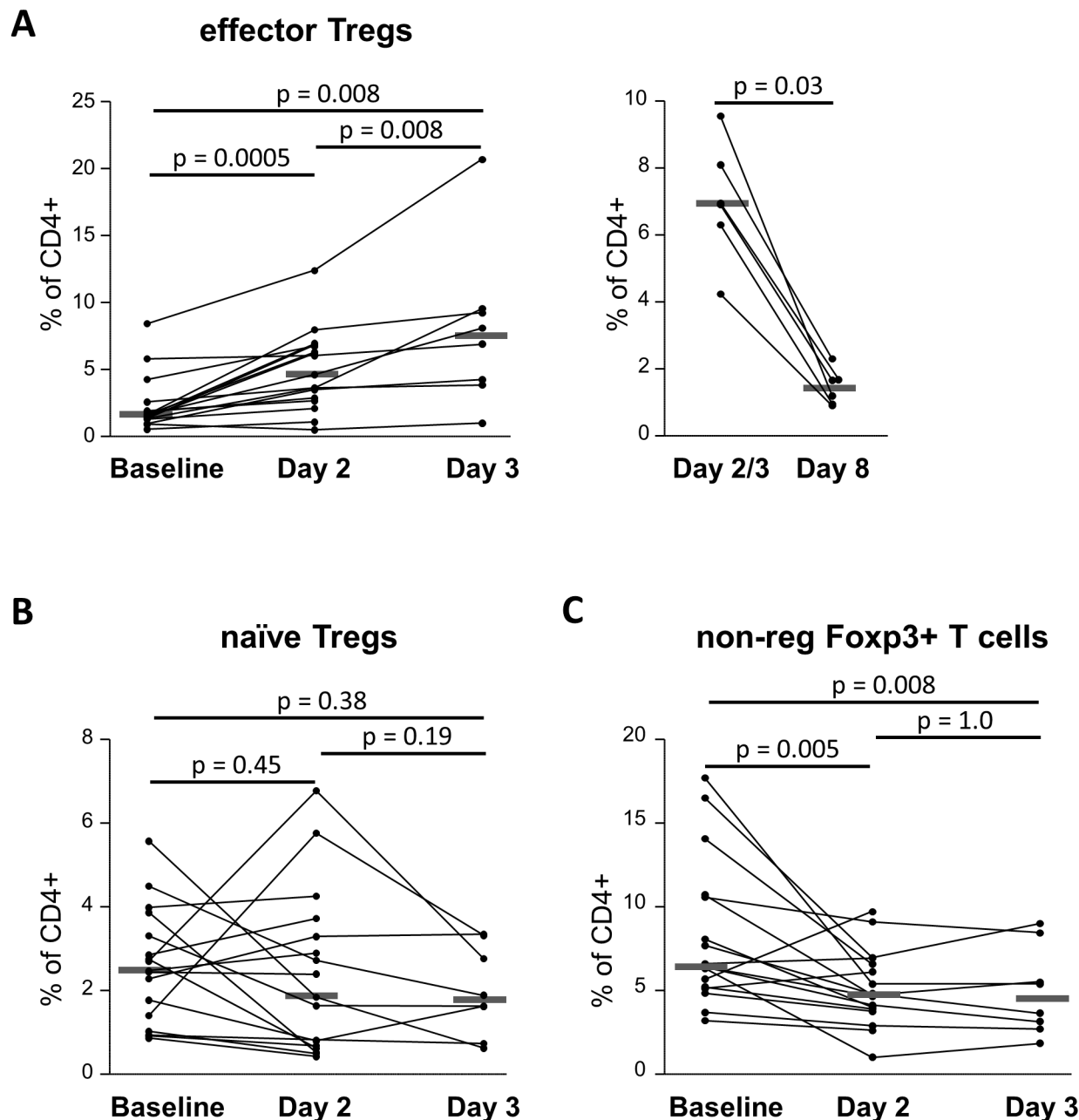


Fig 2. Frequencies of CD4⁺FoxP3⁺ T cell subsets following IV methylprednisolone pulses in SLE patients. Fresh PBMCs from SLE patients were analyzed by flow cytometry as described in Fig 1. Kinetics of (A) effector Tregs, (B) naïve Tregs and (C) non-regulatory FoxP3⁺ T cells were performed in patients with active SLE undergoing IV high dose MP pulse treatment at baseline. Seventeen patients at baseline and day 2, 8 patients at day 3 and 6 patients at day 8 were assessed. (A) Right panel, eTreg cells frequencies at day 3 are displayed (except for two patients for whom these data are missing; eTreg cells frequencies at day 2 are shown instead). Each dot represents an individual assessed in an independent experiment, and the grey bar shows median values. Statistical analyses were performed using the Wilcoxon matched pairs signed ranks test.

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on Treg cells. In the present study, we show that exposure to IV high dose MP pulses induced a marked eTreg increase and a rapid clinical improvement in almost all SLE patients studied. We also showed that a good clinical outcome after one year of follow up was associated with a higher frequency of eTregs at day 2. It can therefore be envisaged that the expansion of eTregs

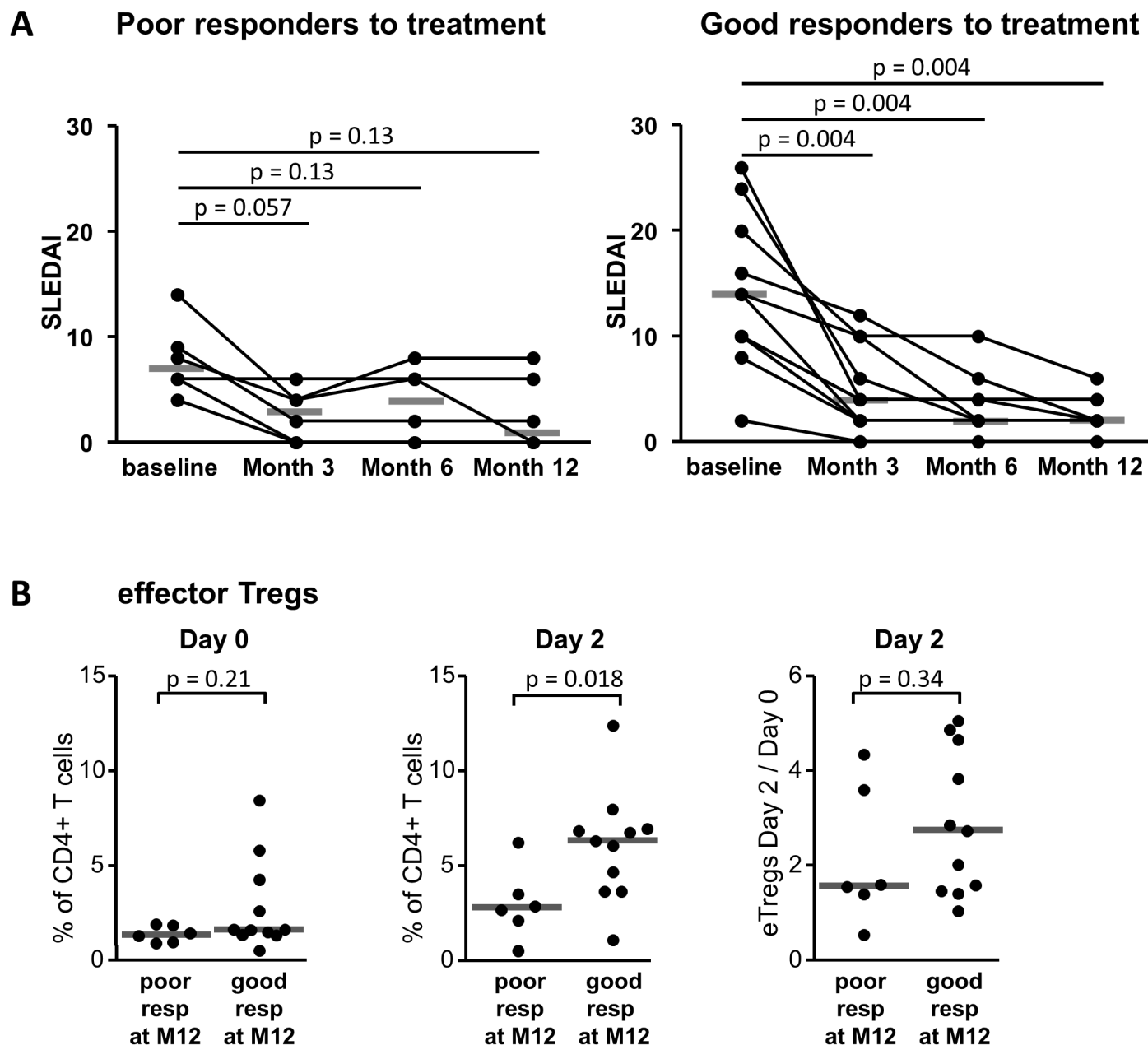


Fig 3. Frequencies of eTreg cells in SLE patients according to their clinical response following IV methylprednisolone pulses. (A) Evolution of the SLENA-SLEDAI score at baseline, month 3, 6 and 12 following IV MP pulses according to the responder status of the patients defined after 12 months of follow-up. (B) Effector Treg frequencies at day 0 (baseline) and day 2 and the ratio of effector Tregs between day 2 and day 0 in poor and good responders after 12 months of follow-up. (A-B) Each dot represents an individual and lines show median values. Statistical analyses were performed using the Wilcoxon matched pairs signed ranks test (A) and the Mann-Whitney *U* test (B).

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plays a role in the anti-inflammatory and immunosuppressive effect of glucocorticoids, especially for the high concentrations achieved by parenteral administration.

The influence of glucocorticoid therapy on regulatory T cells is still a matter of debate. Several studies have shown that glucocorticoid drugs increase *in vitro* and *in vivo* FoxP3 expression and could induce the expansion of CD4⁺CD25⁺FoxP3⁺ regulatory T cells *in vivo* [13, 24–31, 38]. These conclusions have been challenged by two other studies [39, 40]. Sbiera *et al.*

showed that short-term glucocorticoids have little impact on the relative frequency of circulating Treg cells in patients without an inflammatory disease and in mice receiving high-dose IV prednisolone [39]. In this study, Tregs were monitored 14 days after treatment. Here, on the contrary, we clearly showed a dramatic expansion of peripheral Treg cells after IV high dose MP in active SLE. The fact that this expansion was early (i.e., seen as soon as 24 hours after the first pulse) and transient (i.e., not present after 8 days) certainly explains the discrepancy between the two studies. It is also possible that the steroid effect observed on Tregs is disease-dependent, as CD4⁺ T cell homeostasis is markedly perturbed in Lupus patients. Indeed, we did not observe such dramatic eTreg expansions in the steroid-treated patients suffering from other autoimmune diseases that we could study (Sarcoidosis (n = 6), vasculitis (n = 2), myositis (n = 2), data not shown). In transplanted patients treated with MP for acute kidney rejection, Seissler *et al.* observed an increase in a specific subset of Treg cells that displayed the CD4⁺ FoxP3⁺ CD127^{low} CD45RA⁻ HLA-DR⁺ phenotype [40], but they did not observe a global Treg increase. The reported results may be explained by the concomitant use of several immunosuppressors such as cyclosporine A that might have compromised Treg cell proliferation [41, 42]. As the increase in the proportion of eTregs among CD4 T cells corresponds to a significant increase in the eTreg counts, we can exclude that the over-representation of eTregs among CD4⁺ T cells would be solely explained by the selective cell death of CD25⁻ FoxP3⁻ CD4⁺ T cells [43]. Steroid-induced apoptosis [22] is nevertheless an important mechanism that should be taken into account. Indeed, the non-regulatory Foxp3^{low} T cell decrease we observed could be related to apoptosis. However, this interpretation remains speculative, as we could not detect Annexin V⁺ non-regulatory Foxp3^{low} T cells, possibly due to their rapid clearance from the circulation (data not shown).

The effects of steroids are dependent on their binding to the cytoplasmic glucocorticoid receptor (GCR) and their translocation to the nucleus. In the nucleus, the steroid-GCR complex acts as a transcription factor that enhances the expression of numerous genes [44]. Because GCR has been described as an enhancer for FoxP3 gene expression [45], it is highly likely that the eTreg cell expansion that follows the MP pulse in SLE is the consequence of the enhancement of FoxP3 gene expression. However, whether the increase in eTregs is a result of higher FoxP3 expression that would drive FoxP3⁻ and FoxP3^{low} cells to FoxP3^{high} or is a consequence of the proliferation of pre-existing eTreg cells cannot be concluded from the present data. Experiments in mice tracking these different subsets and analysis of the T-cell receptor repertoire of regulatory T cells could help resolve this question.

Because Treg cells are instrumental for the maintenance of self-tolerance, several groups have attempted to manipulate Treg cells *in vitro* or *in vivo* to treat autoimmune diseases [37]. In this regard, Treg transfer therapy is expected to be an efficacious way to prevent organ rejection, graft versus host disease or autoimmune disorders. Different animal models have indicated that the infusion of Treg cells in SLE prone mice was efficient in preventing the development of diseases [46, 47]. In the (NZB×NZW)F1 lupus prone mouse, the progression of nephritis was delayed by the transfer of Tregs from young F1 mice. As Treg transfer therapy has not yet proven its efficiency in human SLE, an alternative strategy is to increase Treg cells *in vivo*. We have shown here that high dose IV MP *per se* is sufficient for the expansion of eTreg cells. High dose IV methylprednisolone is still of value in treating patients with SLE whose disease is not controlled. It provides symptomatic relief and the regression of severe forms of disease and may also enable a reduction in the dose of maintenance glucocorticoids [18, 19]. The high concentrations of glucocorticoids achieved by IV pulse appears to have a more immediate and profound effect on Treg cells compared to conventional oral treatment [48]. However, despite the clinical efficacy of pulse for the initial management of active SLE, this benefit is not maintained for prolonged periods [17, 49]. This has to be put into perspective

with the observation that eTreg cell expansion is only transient. Because repeated use of high dose IV MP is limited by the increased risk of infection [18, 19, 50], alternative strategies have to be developed to sustain Treg cell expansion over the long term. One of these strategies is to increase Treg cells *in vivo* using Treg cell related cytokines such as IL-2. Three phase I/II trials have evaluated the effects of IL-2 in chronic GVH, type 1 diabetes and HCV related infectious cryoglobulinemic vasculitis [51–53]. While IL-2 was shown to significantly increase the proportion of circulating Treg cells in all studies, this cytokine was also shown to ameliorate skin involvement in GVHD as well as in vasculitis, but was inefficient in type 1 diabetes.

The use of IL-2 in SLE seems particularly interesting because a reduction of this cytokine induced an imbalance of Treg cells and effector T cells leading to accelerated disease progression in the lupus prone mouse [54]. Administration of IL-2 promotes the homeostatic proliferation of endogenous Tregs, delays progression of the disease [54, 55] and corrects regulatory T cell defects in patients SLE [56]. A case of a patient with refractory SLE successfully treated with IL-2 has been reported recently [57]. Due to their synergistic effect, the combination of glucocorticoids and IL-2 also seems particularly interesting [58].

Our results should nevertheless be interpreted with a few limitations in mind. It is important to underline that the clinical outcome depends also on the maintenance of remission and probably not only on a transient increase in Tregs. Furthermore, one cannot discern with certainty whether all Treg cells need to expand to elicit a clinical response. It could be possible that the expansion of specific clones is the determining factor to control autoimmunity. In addition, a change in Tregs in the peripheral blood cannot predict the action of Treg cells at the site of inflammation and the level of regulation that they exert there. It was also demonstrated by others that the increase in Treg cells associated with SLE remission seems to be independent of the treatment used [13]. However, the increase in effector Treg cells that we observed shortly after the MP pulses is more in favor of a direct impact of glucocorticosteroids on the regulatory T cell physiology rather than a consequence of disease remission and/or the concomitant use of other immunosuppressive drugs. We showed that expanding eTreg cells upon high dose IV MP led to CD15s expression, a marker highly specific for activated, terminally differentiated, and mostly suppressive eTreg cells which enabled us to conclude that IV MP induces bona fide regulatory T cells [36]. However, we did not study other known markers for Tregs such as CD127 [59] and Helios [60, 61] which have already been evaluated in lupus [62–65] or new markers such as TIGIT [66] or FCRL3 [67]. It might be worthwhile to include these markers in further studies to better characterize these cells.

Altogether, we showed that high dose IV MP leads to a rapid, marked and transient increase in circulating effector Treg cells in most patients with active SLE. This was associated with a clinical improvement immediately after the MP pulses. Importantly, most of the patients with a better Treg expansion did not experience new flares after 1 year of follow-up. This indicates that high dose IV MP may prevent flares by increasing the number of eTreg cells. We therefore suggest that therapeutic strategies aimed at maintaining expanding Treg cells over the long term may help to prolong the beneficial effect of MP IV pulses. Our results also strengthen the rationale for the therapeutic use of Treg cells in the prevention of flares in autoimmune diseases with a remitting-relapsing course. Due to the limited sample size of our study group, additional evidence should be gathered in SLE patients.

Supporting Information

S1 Fig. Numbers of circulating effector Tregs before and after IV high dose methylprednisolone in SLE patients. The frequency of eTreg were determined by flow cytometry as shown in Fig 1. The absolute counts for circulating eTreg cells were calculated by multiplying

the frequencies of eTreg cells among CD4⁺ T cells with the frequencies of CD4⁺ T cells among total lymphocytes and the total lymphocyte blood count which was available at day 3 only for 10 patients. Each dot represents an individual assessed in an independent experiment, and the grey bar shows median values. Statistical analyses were performed using the Wilcoxon matched pairs signed ranks test.

(TIF)

S2 Fig. CD15s expression in CD4⁺FoxP3⁺ T cell subsets following IV high dose methylprednisolone pulses in SLE patients. Fresh PBMCs from SLE patients were analyzed by flow cytometry, gated on CD4⁺ T lymphocytes, for the expression of FoxP3 and CD15s before IV high dose MP pulses i.e., day 0 and at day 2 after the first pulse. Percentages of the different subsets are shown. Representative analyses from one SLE patient are shown (pt #15).

(TIF)

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Author Contributions

Conceived and designed the experiments: AM RJ GG ZA LM MM. Performed the experiments: RJ DC JF LC. Analyzed the data: AM RJ FCA JH ZA MM. Contributed reagents/materials/analysis tools: AM RJ GG ZA LM MM. Wrote the paper: AM RJ LM GG ZA MM. Wrote the draft and the final version of the manuscript: AM RJ LM GG ZA MM Recruited patients: AM FCA JH ZA MM.

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