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Immunophenotypic Analysis Reveals Differences in Circulating Immune Cells in the Peripheral Blood of Patients with Segmental and Nonsegmental Vitiligo

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Accumulating studies have indicated immune-based destruction of melanocytes in both segmental vitiligo (SV) and non-SV (NSV). Whereas SV often occurs unilaterally during childhood and stabilizes after an initial period of activity, the disease course of NSV is usually slowly progressive, with new lesions occurring bilaterally during life. This suggests an involvement of distinct pathophysiology pathways, specifically increased systemic immune activation in patients with NSV but not in patients with SV. This research aimed to identify the differences in immune cells in the blood of patients with SV and NSV through immunophenotyping of circulating cells. Regulatory T cells were unaffected in patients with SV compared with that in healthy controls but decreased in patients with NSV. In patients with NSV, the reduction in regulatory T cells was associated with the presence of other systemic autoimmune comorbidities, which were less present in SV. Similarly, the absence of a melanocyte-specific antibody response in patients with SV suggests less involvement of B-cell immunity in SV. These data show that in contrast to patients with NSV, no increased systemic immunity is found in patients with SV, indicating that SV pathogenesis is associated with a localized cytotoxic reaction targeting epidermal melanocytes.

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INTRODUCTION

Vitiligo is the most common skin depigmenting disorder characterized by white patches resulting from the loss of pigment-producing cells, melanocytes (Bergqvist and Ezzedine, 2020). It affects approximately 0.5% of the general population with no apparent differences in rates of occurrence according to sex, skin type, or ethnicity (Boniface et al., 2018). An international consensus classified vitiligo into two subtypes (Ezzedine et al., 2012). The commonest form, nonsegmental vitiligo (NSV), shows symmetrical depigmentation of the body. Contrary, segmental vitiligo (SV) is less common (±10%) and is characterized by a unilateral distribution. In addition, NSV shows an unpredictable disease course, whereas SV typically stabilizes a few months after onset. Altogether, this suggests that distinct pathophysiology pathways might be involved, which could clarify the differences in clinical presentation and disease course.

Initially, somatic mosaicism, neurogenic mechanisms, and oxidative stress were suspected to be the underlying cause of SV (Speeckaert et al., 2020). Only recently, immune-mediated pathophysiology of SV has been recognized. Increasing evidence has shown immune-based cytotoxic destruction of melanocytes in SV, with lesional IFN-γ–producing melanocyte antigen–reactive $CD8^{+}$ T-cell infiltrates migrating to the basal layer (Attili and Attili, 2013; Shin et al., 2016; van Geel et al., 2010). Although NSV is closely associated with other autoimmune disorders, for example, thyroid disease and alopecia areata, systemic autoimmune comorbidities are less common in patients with SV (Dahir and Thomsen, 2018; Speeckaert et al., 2020). Nevertheless, localized skin inflammation, for example, linear morphea, is repeatedly observed in patients with SV, implying a local inflammatory response (Speeckaert et al., 2020). The comparison between SV and NSV provides a unique setting to study whether antimelanocyte immunity remains localized in SV and spreads systemically in NSV.

A study on gene expression profiles of patients with SV and NSV and of healthy individuals showed that differentially expressed genes in SV were involved in the adaptive immune response, whereas in NSV, the regulation of the innate immune response and B-cell differentiation and activation was more prominent, implying that SV and NSV may utilize different immune responses and mechanisms for melanocyte destruction (Wang et al., 2016). Concomitantly, the blood of

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Abbreviations: cTfh, circulating T follicular helper; NSV, nonsegmental vitiligo; SV, segmental vitiligo; Treg, regulatory T cel; TYR, tyrosinase

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Table 1. Patient Characteristics

	Healthy Donor			SV			NSV			
	n	%	IQR/SD	n	%	IQR/SD	n	%	IQR/SD	*P*-Value[1]
Total	22			12			22			
Age										
<25	2	9.1		3	25		1	4.5		
25–50	17	77.3		8	66.7		14	63.6		
>50	3	13.6		1	8.3		7	31.8		
Mean	36		(23–48)	34		(22–46)	43		(29–57)	>0.05
Sex										>0.05
Male	6	27.3		9	75		11	50		
Female	16	72.7		3	25		11	50		
Skin type[2]										>0.05
Type 1	0			0			1	4.5		
Type 2	13	59.1		8	66.7		12	54.5		
Type 3	7	31.9		3	25		8	36.4		
Type 4	1	4.5		1	8.3		0			
Type 5	1	4.5		0			1	4.5		
Type 6	0			0			0			
Comorbidities										
Thyroid disease				0			3	13.6[3]		
Alopecia Areata				1	8.3		0			
DM type 1				0			4	18.2[3]		
RA				0			0			
SLE				0			0			
Psoriasis				0			0			
Other[4]				0			1	4.5[3]		
Vitiligo age of onset (y) mean				25		±14.8	33		±15.6	>0.05
Disease duration (y) median				4		(2.3–12.3)	8	(4–12.5)		>0.05
% Affected body surface area median				0.75		(0.5–1.9)	1	(0.9–3.9)		>0.05

Abbreviations: CREST, calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; DM, diabetes mellitus; IBD, inflammatory bowel disease; IQR, interquartile range; NSV, nonsegmental vitiligo; PMR, polymyalgia rheumatica; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SV, segmental vitiligo.

[1]*P*-value tested with Student's *t*-test or Mann–Whitney test.

[2]Skin type according to the Fitzpatrick skin scale.

[3]One patient with NSV showed multiple autoimmune comorbidities (hypothyroidism, DM type 1, and colitis ulcerosa).

[4]Other specified, including Addison's disease, arthritis psoriatica, autoimmune hepatitis, IBD, celiac, CREST, morphea, pernicious anemia, PMR, sarcoidosis, scleroderma, Sjogren's Syndrome.

patients with NSV showed a decrease in regulatory T cells (Tregs) and an increase in unswitched memory B cells compared with healthy control blood, which was related to disease activity (Raam et al., 2018). Considering the positive correlation between switched memory B cells and circulating T follicular helper (cTfh) cells, patients with active NSV show activation of germinal centers and faster B-cell isotype switching (Raam et al., 2018). Despite data showing involvement of B cells and germinal center reactions, the correlation to a humoral response remained unstudied.

To our knowledge, analysis of circulating immune cells, involvement of a humoral response, and germinal center reactions in SV have not been fully characterized. Whereas associated autoimmune diseases are more common in NSV (Alkhateeb et al., 2003; Gill et al., 2016; Hadi et al., 2020; Spritz and Andersen, 2017), evidence points to a temporary cytotoxic response targeting melanocytes in SV, suggesting differences in systemic immune cell dysregulation between patients with SV and those with NSV.

This study aimed to compare the differences in cellular and humoral adaptive immunity and innate immunity in human blood of patients with SV and NSV that can contribute to clinical presentation and disease progression. Our results show no increased systemic immunity in patients with SV, in contrast to that in patients with NSV, and points to localized immune-based cytotoxic destruction of melanocytes.

RESULTS

Demographics and clinical characterization of study subjects

The characteristics of the participants are shown in Table 1. Patients with SV had an average age of 34 years, which is higher than the average age of the general SV population because of the age ≥18 years inclusion criterion. SV had stabilized in our patient cohort, for at least 12 months. To focus our comparative study on the differences between SV and NSV without the interference of active versus stable vitiligo disease activity, we compared stable patients with SV with stable patients with NSV. The majority of included patients with SV had type-2 skin, and only one patient had alopecia areata as autoimmune comorbidity. Patients with NSV had stable disease and an average age of 43 years; the majority of patients had a type-2 or -3 skin type, and 6 of the 22 patients (27.2%) showed autoimmune comorbidities. Patients

with SV and NSV did not differ in age of onset, disease duration, and affected body surface area. Healthy controls were comparable with regard to age, sex, and skin type with the patients with vitiligo.

Patients with NSV with secondary autoimmune comorbidities have less circulating Tregs

Perturbations in Treg numbers and function in vitiligo remain indistinct. Some studies report systemically reduced Tregs in NSV (Ben Ahmed et al., 2012; Dwivedi et al., 2013; Giri et al., 2020; Hegab and Attia, 2015; Lili et al., 2012; Raam et al., 2018), whereas others show that abundance of Tregs in NSV trends toward an increase (Abdallah and Saad, 2009; Moftah et al., 2014). Moreover, Treg involvement in SV remains unstudied. Therefore, we studied systemic Treg and type-1 Treg numbers and IL-10 production in our cohort of patients with SV and NSV and in healthy donor samples. The gating strategy for Tregs is depicted in Supplementary Figure S1. Circulating Tregs were significantly decreased in the blood of patients with NSV compared with the blood of those with SV and those of healthy controls (Figure 1a). However, IL-10 production by Tregs, measured as IL-10^+ cells after intracellular FACS staining, was not affected in these patients (Figure 1a). The level of Tregs in patients with SV did not differ from that in healthy controls (Figure 1a). We verified these findings on the RNA expression level and in an independent patient cohort using the RNA-sequencing dataset from Wang et al. (2016). This dataset contains 20 patients with SV, 20 patients with NSV, and 20 healthy control individuals (within each patient group, 5 patients were pooled into a new sample, giving a total of 4 samples) (Wang et al., 2016). Gene expression profiles of patients with NSV and SV and of healthy individuals were then analyzed for the presence of a Treg gene expression signature. For this, we made use of a gene signature used to discriminate between Tregs ($CD25^{high}$) and conventional $CD4^+$ T cells ($CD25^-$) (Niedzielska et al., 2018). This gene signature comprises 25 genes, of which 21 genes are upregulated and 4 genes are downregulated on Tregs compared with those on conventional $CD4^+$ T cells (Supplementary Table S1). Similar to cellular Treg analysis, patients with NSV showed decreased expression of the Treg signature compared with healthy individuals ($P < 0.05$) (Figure 1b). Because of the large spread among SV samples, comparing Treg levels in patients with NSV and in those with SV did not reach significance ($P = 0.08$) (Figure 1b).

Because systemic autoimmune comorbidities might influence circulating Treg numbers, we compared Treg levels in patients with NSV with or without comorbidities as indicated in Table 1. Patients with NSV with autoimmune comorbidities showed significantly fewer Tregs than patients with NSV without autoimmune comorbidities (Figure 1c), suggesting that impaired Treg numbers might be the consequence of secondary autoimmune responses and not specific to vitiligo pathogenesis. Treg levels in patients with NSV without autoimmune comorbidities still trended toward a decrease compared with those in healthy controls, but this did not reach significance in our patient cohort size ($P = 0.09$; data not shown). Nevertheless, gene expression patterns of patients with NSV showed a less pronounced Treg signature, even when autoimmune conditions were absent (the patient cohort of Wang et al. [2016] only includes patients with no history of any other autoimmune condition) (Figure 1b).

In contrast to Tregs, the percentage of type-1 Tregs did not demonstrate significant differences between the studied groups; nonetheless, IL-10–producing type-1 Tregs were increased in patients with SV (Figure 1d). To conclude, these results further support that Tregs but not type-1 Tregs are negatively affected in (some) patients with NSV, which may facilitate the development of autoimmune comorbidities. In contrast, Tregs remain unaffected in patients with SV, consistent with less systemic autoimmune comorbidities in patients with SV.

Antibody responses against melanocyte antigens are present only in patients with NSV

Antibody responses against melanocyte antigens, for example, tyrosinase (TYR) and TRP-2, have been found in the sera of patients with NSV (Kemp et al., 2007). However, sera from patients with SV have rarely been tested for the presence of autoantibodies. To test reactivity to melanocyte antigens in patients with SV and NSV, sera were evaluated for antibody reactivity against TYR, TYRP1, TYRP2, PMEL, TYR hydroxylase, MART-1, and MCHR1. Antibodies against selected melanocyte antigens were present in the circulation of a significant proportion of patients with NSV (Figure 2). In total, 8 of 22 (36%) patients with NSV were found to have antimelanocyte antibody responses (Supplementary Table S2). Moreover, three of eight patients showed antibody reactivity to several melanocyte antigens (Supplementary Table S2). Patients with NSV with antibody responses against the antigens mentioned earlier were not significantly different from patients with NSV without autoantibodies regarding age, sex distribution, skin type, presence of secondary autoimmune comorbidities, vitiligo age of onset, disease duration, and percentage of affected body surface area (Supplementary Table S3). Contrary, none of the patients with SV showed an antibody response (Figure 2), indicating that the presence of a humoral immune response to melanocyte antigens is limited to patients with NSV.

Patients with SV have fewer circulating antibody-producing plasmablasts

We subsequently analyzed whether the absence of antibody responses in SV is also reflected in less B-cell activation and plasma-cell differentiation. The gating strategy for B cells is depicted in Supplementary Figure S1. The percentage of total B cells ($CD3^-$ $CD19^+$) was comparable between patients with vitiligo and healthy individuals (Figure 3a). Similarly, naive B cells and unswitched and switched memory B cells did not demonstrate significant differences between the studied groups (Figure 3a), implying that B cells seem to mature similarly in patients with vitiligo and in healthy individuals. Similarly, transitional B cells did not differ between patients with SV and NSV nor the patients and the healthy controls (Figure 3b). However, plasmablasts were significantly decreased in blood from patients with SV compared with healthy donor blood (Figure 3c). Concomitantly, plasmablasts showed a trend toward a decrease in patients with SV compared with that in patients with NSV, suggesting that

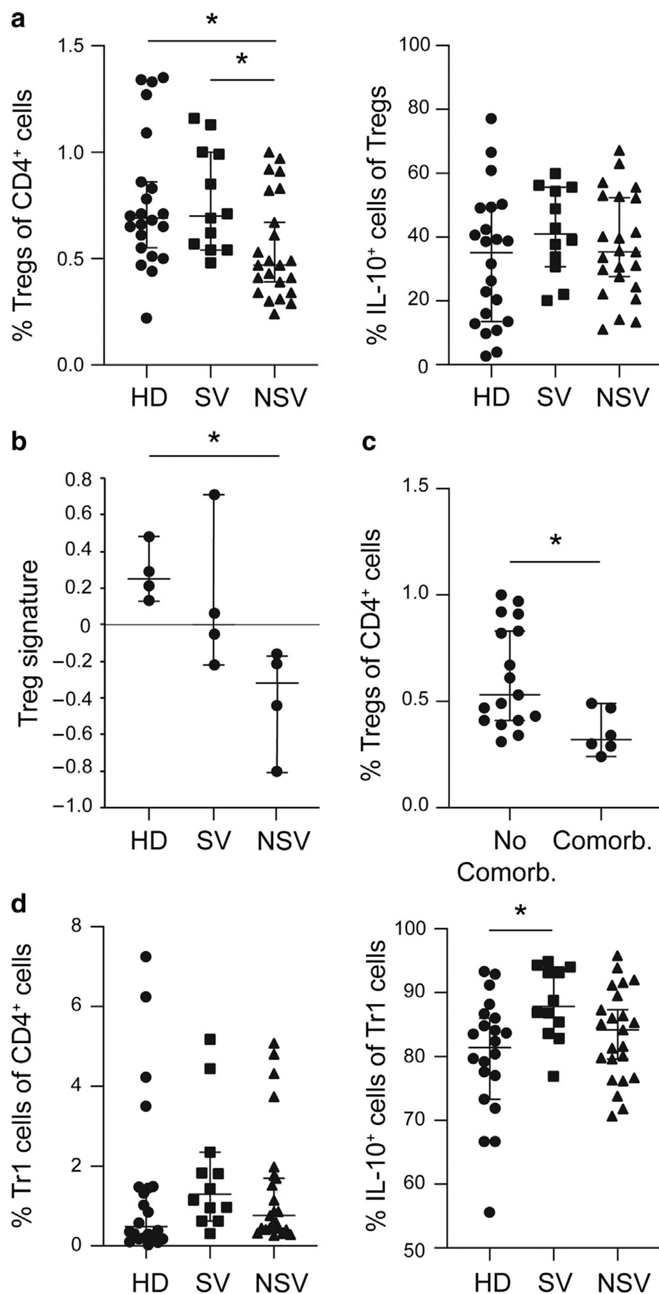

Figure 1. Comparison of circulating Treg subpopulations in patients with vitiligo (**a**) The percentage of Tregs among $CD4^+$ T cells (left) and IL-10–producing cells among Tregs (right) in HD (n = 22), patients with SV (n = 12), and patients with NSV (n = 20). (**b**) Expression of the Treg core gene signature in HD (n = 20), patients with SV (n = 20), and patients with NSV (n = 20) (within each group, five samples were pooled into a new sample). The genes included in this Treg core signature are included in Supplementary Table S1. (**c**) The percentage of Tregs among $CD4^+$ T cells in patients with NSV without (no Comorb., n = 16) or with comorb. (n = 6). (**d**) The percentage of Tr1 among $CD4^+$ T cells (left) and IL-10–producing cells among Tr1 (right) in HD, patients with SV, and patients with NSV. Results are shown as individual dot plots with a line as median and 95% CI for **a**, **c**, and **d** or median and minimum and maximum for **b**. ANOVA and Student's *t*-test are significant as indicated; $*P < 0.05$. CI, confidence interval; Comorb, autoimmune comorbidity; HD, healthy control; NSV, nonsegmental vitiligo; SV, segmental vitiligo; Tr1, type-1 regulatory T cell; Treg, regulatory T cell.

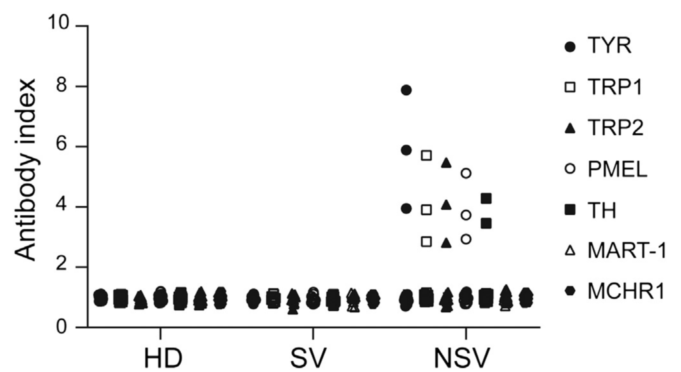

Figure 2. Presence of melanocyte-specific antibody responses in patients with vitiligo. Sera of HD (n = 30), patients with SV (n = 12), and patients with NSV (n = 20) were analyzed in radio-binding assays for the presence of antibodies against TYR, TRP1, TRP2, PMEL, TH, MART-1, and MCHR1. The antibody index for each individual patient is shown and is calculated as c.p.m. immunoprecipitated by tested serum divided by the mean c.p.m. immunoprecipitated by the group of HD sera. Each serum was tested in at least three independent experiments. Next, the mean antibody index was calculated from these values. Patient sera with an antibody index above the upper limit of normal (mean antibody index + 3 × SD of the HD individuals) were regarded as positive for antibody reactivity. c.p.m., count per minute; HD, health control; NSV, nonsegmental vitiligo; SV, segmental vitiligo; TH, tyrosinase hydroxylase; TYR, tyrosinase.

patients with SV have fewer circulating antibody-producing cells.

Patients with SV have more cTfh2 and cTfh17 cells but no increase in the number of active cTfh cells

Our data so far indicate that patients with SV have no melanocyte-specific antibody response and a diminished humoral response, illustrated by fewer circulating plasmablasts (Figures 2 and 3c). To verify whether this reduced plasmablast differentiation stems from reduced germinal center help, we analyzed the presence of cTfh cells. Levels of both cTfh cells and active cTfh cells ($PD-1^+$ $ICOS^+$) were unaffected in patients with NSV compared with that in healthy control individuals. Instead, the number of cTfh cells were significantly increased in patients with SV compared with that in patients with NSV (Figure 4a). Similarly, the number of cTfh cells with an active phenotype were increased in patients with SV compared with those in both patients with NSV and healthy controls ($P < 0.05$) (Figure 4a).

On the basis of the expression of CXCR3 and CCR6, cTfh cells can be classified into cTfh1, cTfh2, and cTfh17 cells (Supplementary Figure S1) (Koutsakos et al., 2019). These cells differ in their ability to provide help to naive and memory B cells, and thus the abundance of specific cTfh cell subsets might be as important as total cTfh cell levels. In blood, cTfh1 cells did not show significant differences between the studied groups (Figure 4b). Contrary, cTfh2 and cTfh17 cells, which are superior IL-21 producers to cTfh1 cells and especially provide help to naive B cells, were significantly increased in patients with SV compared with those in patients with NSV and in healthy controls ($P < 0.05$) (Figure 4b). More importantly, patients with SV did not show an increase in active cTfh2 and cTfh17 cells (Figure 4c).

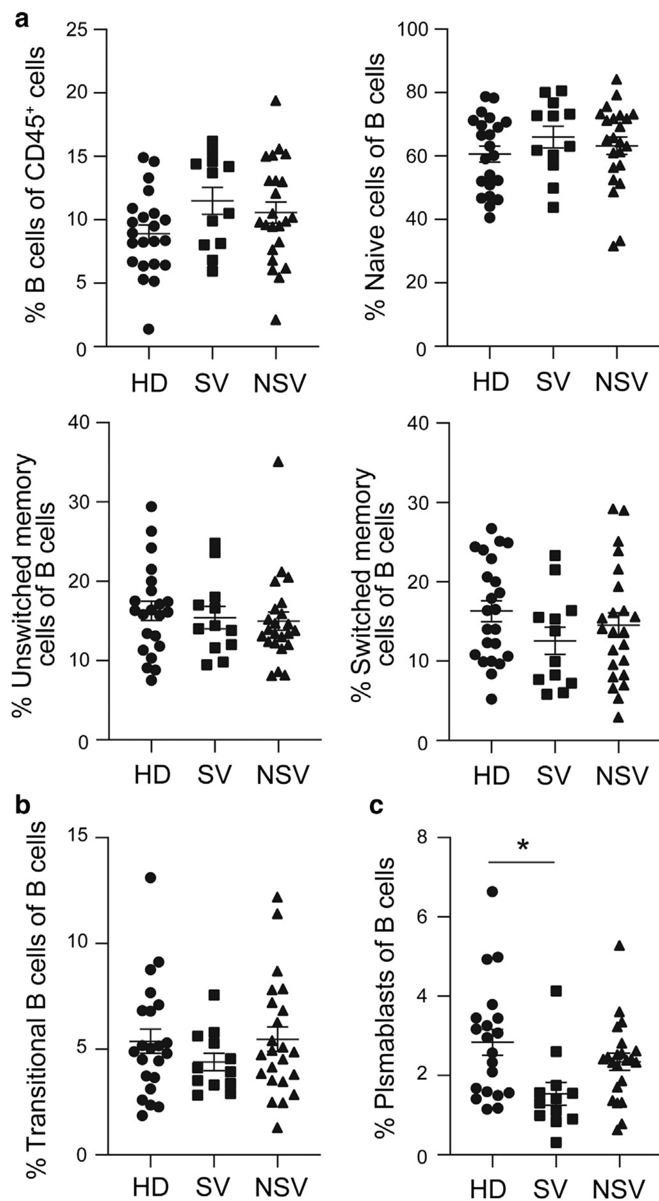

Figure 3. Distribution of peripheral B-cell subsets in patients with vitiligo. (a–c) The percentage of B cells among lymphocytes, naive B cells, unswitched memory B cells, and switched memory B cells among (a) B cells, (b) transitional B cells, and (c) plasmablasts among B cells in HD (n = 22), patients with SV (n = 12), and patients with NSV (n = 20). Results are shown as individual dot plots with means ± SEM. ANOVA test is significant as indicated; *$P < 0.05$. HD, health control; NSV, nonsegmental vitiligo; SV, segmental vitiligo.

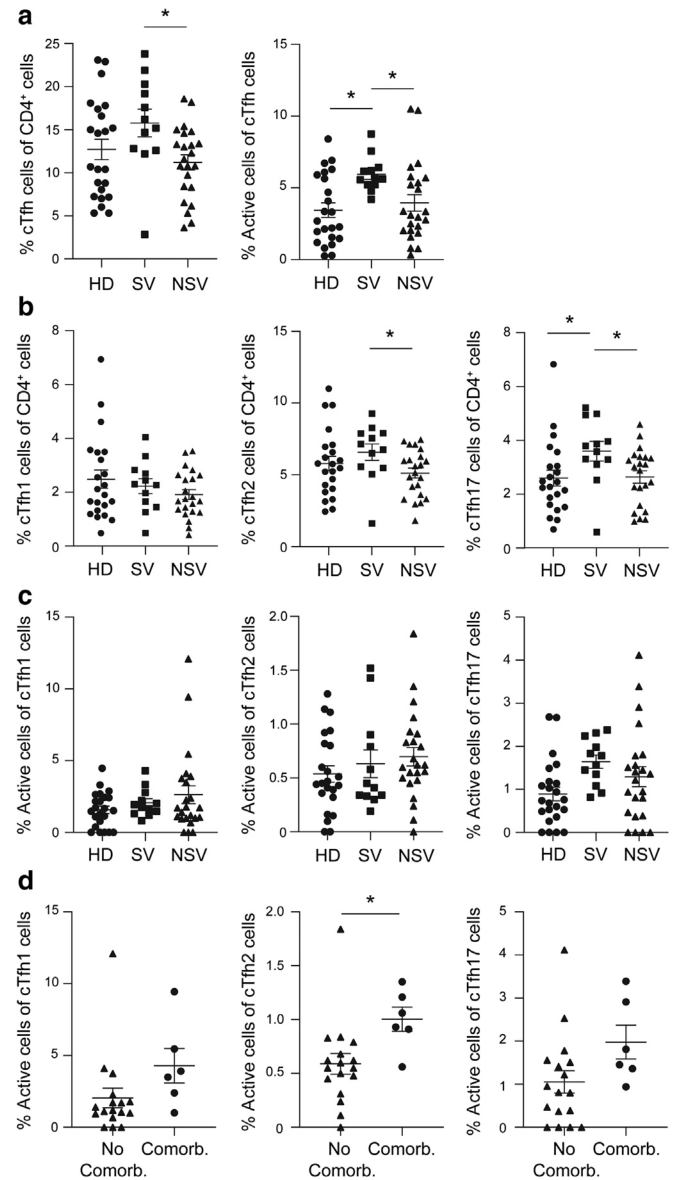

Figure 4. Comparison of cTfh cell subsets in patients with vitiligo. (a–c) The percentage of cTfh cells among (a) $CD4^+$ cells and active ($PD\text{-}1^+$ $ICOS^+$) cTfh cells, (b) cTfh cell subsets and active cells within $CD4^+$ cells, and (c) cTfh cell subsets in HD (n = 22), patients with SV (n = 12), and patients with NSV (n = 20). (d) The percentage of active cells among cTfh cell subsets in patients with NSV without (no comorb., n = 16) or with Comorb. (n = 6). Results are shown as individual dot plots with means ± SEM. ANOVA and Student's *t*-test are significant as indicated; *$P < 0.05$. Comorb., autoimmune comorbidity; cTfh, circulating T follicular helper; HD, health control; NSV, nonsegmental vitiligo; SV, segmental vitiligo.

Concomitantly, active cTfh cell subsets did not differ between patients with NSV and healthy individuals (Figure 4c). Because the provision of B-cell help is limited to activated cTfh cells and the percentage of active cTfh1, cTfh2, and cTfh17 cells do not differ between SV and NSV, it seems that both vitiligo cohorts show normal germinal center help that is not different from that of the healthy controls.

As for Treg numbers, systemic autoimmune comorbidities might affect cTfh cell levels and activation status. NSV is closely associated with other autoimmune conditions, whereas these are less common in patients with SV (Dahir and Thomsen, 2018; Speeckaert et al., 2020). These comorbidities may involve humoral responses and increased B-cell help from cTfh cells. Indeed, when patients with NSV were divided into subgroups according to the presence of secondary autoimmune comorbidities, it was evident that systemic autoimmune comorbidities were associated with increased percentages of active cTfh2 cells ($P < 0.05$) and cTfh17 cells ($P = 0.07$) but not with cTfh1 cells (Figure 4d). Collectively, our results show increased cTfh2 and cTfh17 cell numbers in SV but no increased germinal center reactions in human vitiligo. Consistent with an increase in activated cTfh2 and cTfh17 cell subsets in type-I diabetes and

thyroid disease (Gensous et al., 2018), enhanced cTfh cell activation in patients with NSV with secondary autoimmune comorbidities seems to result from those rather than from skin autoimmunity.

Patients with vitiligo do not differ from healthy controls with regard to circulating NK cells

Besides aberrations in the adaptive immune response, innate immunity is suggested to be involved in NSV pathogenesis as well but left unstudied in patients with SV. The gating strategy for NK cells is depicted in Supplementary Figure S1. Systemic NK cell levels were not different in both vitiligo subtypes compared with that in healthy control blood (Figure 5a). Expression of the activating NK cell receptor, NKG2D, on NK cells and cytotoxic CD56^{dim} NK cells was significantly decreased in patients with NSV compared with that in healthy controls but unaffected on cytokine-producing CD56^{bright} NK cells (Figure 5b). However, in the blood of patients with SV, no difference in NKG2D expression was seen compared with that in the blood of healthy controls. Because NKG2D can be expressed by CD8$^+$ T effector memory cells and because an increased expression has been observed in active NSV skin (Jacquemin et al., 2020), we analyzed NKG2D expression by peripheral CD3$^+$ T cells. No significant differences in the proportion of NKG2D$^+$ CD3$^+$ T cells were seen in the blood of patients with NSV compared with that in the blood of patients with SV and healthy controls (data not shown). Because NK cells were unaffected in patients with SV, innate immunity seems to be less involved in SV pathogenesis.

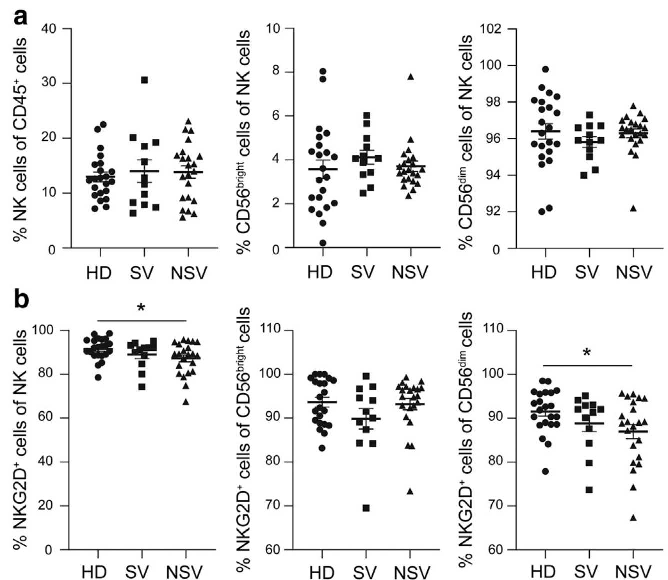

Figure 5. Distribution of circulating NK cells in patients with vitiligo. (**a**) The percentage of NK cells among CD45$^+$ cells, CD56^{bright} cells, and CD56^{dim} cells among NK cells and (**b**) the percentage of NKG2D$^+$ cells among NK cells, CD56^{bright} cells, and CD56^{dim} cells in HD (n = 22), patients with SV (n = 12), patients with NSV (n = 20). (**b**). Results are shown as individual dot plots with means ± SEM. ANOVA tests are significant as indicated; *$P < 0.05$. HD, health control; NSV, nonsegmental vitiligo; SV, segmental vitiligo.

DISCUSSION

This study provides important insights into the differences between SV and NSV pathogenesis and shows that in contrast to NSV, SV does not involve systemic immune activation. We found that Tregs are less abundant in patients with NSV than in healthy controls but did not differ in patients with SV. Furthermore, a humoral response and germinal center reactions were not observed in patients with SV. This is consistent with fewer autoimmune comorbidities in patients with SV and points to a local autoimmune reaction.

Previous studies have shown the presence of melanocyte-specific antibodies in some patients with NSV (Kemp et al., 2007), whereas this remained largely unstudied in patients with SV. This work shows that the involvement of a humoral response against melanocyte antigens is restricted to patients with NSV. The absence of a melanocyte-specific antibody response is consistent with the observation that there is no systemic immune activation in patients with SV. Although autoantibodies were found in some patients with NSV, we hypothesize that this is an underestimation because we tested only seven common melanocyte autoantibody targets. Indeed, in immunoprecipitation experiments with melanocyte extracts, 100% of patients with NSV and 0% of healthy controls were found to have antimelanocyte antibodies in their sera (Naughton et al., 1983). In addition, incidence and level of autoantibodies have been correlated with disease activity and the extent of the disease, meaning that patients with active vitiligo and patients with 5−10% skin depigmentation are more likely to have circulating antimelanocyte antibodies (Harning et al., 1991; Naughton et al., 1986).

Tregs induce anergy in melanocyte-specific T cells in healthy individuals (Maeda et al., 2014). More importantly, it is assumed that melanocyte-reactive CD8$^+$ T cells escape anergy by loss of coinhibitory CTLA-4 expression in NSV. Similarly, we show that Treg numbers are decreased in patients with stable NSV, most prominently in patients with secondary autoimmune comorbidities, as hypothesized earlier (Le Poole and Mehrotra, 2017). Even in the absence of autoimmune comorbidities, we found that patients with NSV still show a trend toward a decrease in Treg numbers and signatures. In a previous study, the abundance and activity of circulating Tregs in patients with NSV were shown to be similar to those in healthy controls but reduced in the skin of patients with NSV, explained by the failure of Tregs to home to the skin in vitiligo (Klarquist et al., 2010). Contrary to our cohort, half of these patients showed progressive disease and were under treatment at the moment of collecting blood samples. Therefore, these results might be explained by different disease activity. This highlights the importance of reporting these patient characteristics in great detail. Because numerous studies have not clearly reported the presence or absence of secondary autoimmune comorbidities or disease activity while studying Tregs in NSV, it is difficult to place results into context because the greatest differences are seen in those with other autoimmunities.

The involvement of NK cells in vitiligo has been suggested by previous studies. RNA analysis of NSV skin biopsies revealed high expression of genes of the innate immune system, especially NK cells, compared with that in healthy skin, and more NK cells were found in both lesional and nonlesional NSV skin (Yu et al., 2012). Similarly, NK cells were shown to be significantly increased in the blood of patients with stable NSV compared with that in healthy

controls (Tulic et al., 2019). However, we together with others (Raam et al., 2018) did not detect increased peripheral NK cell levels. Nevertheless, NKG2D expression by NK cells was shown to be significantly decreased in patients with NSV, especially on CD56^{dim} cells, and *KLRC4-KLRK1*, which encodes NKG2D, was found to be downregulated in the blood of patients with SV (Wang et al., 2016). In lesional NSV skin, the stress molecules MICA/MICB (ligands for the activating NKG2D receptor) were shown to be expressed in dermal areas but not in nonlesional or healthy skin (Raam et al., 2018). In addition, IFN-γ−producing innate lymphoid cells were shown to initially induce CXCR3B-mediated melanocyte apoptosis (Tulic et al., 2019). Recently, increased NKG2D expression was found on skin-resident NK cells, NKT cells, and CD8^{+} effector memory T cells, especially in patients with active disease (Jacquemin et al., 2020). Contrary to increased NKG2D expression by CD8^{+} effector memory T cells in the skin of patients with active NSV, no significant difference in the proportion of these cells was seen in the blood of patients with NSV compared with that of healthy controls (Jacquemin et al., 2020). Therefore, it is suggested that a skin factor (possibly IL-15, IFN-α) is responsible for the promotion of NKG2D expression (Jacquemin et al., 2020). In addition, NKG2D upregulation in vitiligo occurs in response to insults and stress, which primarily occurs in lesional skin (Plaza-Rojas and Guevara-Patiño, 2021). Therefore, the involvement of NK cells in NSV and SV pathogenesis remains indistinct but suggests skin-resident NK cells to be involved in the initial initiation of the antimelanocyte autoimmunity during active disease rather than long-lasting systemic NK cell involvement.

To our knowledge, immunophenotypic analysis of circulating immune cells in the blood of patients with SV, compared with those in patients with NSV and in healthy individuals has not been previously reported in the literature. Our results strengthened the notion that immunity plays an important role in vitiligo pathogenesis. Most importantly, our study highlights the immunological difference between NSV and SV. NSV is characterized by systemic immune activation, decreased Treg levels, and the development of autoimmune comorbidities. In contrast, the absence of systemic immune activation in patients with SV indicates that SV pathogenesis is associated with a localized cytotoxic reaction against epidermal melanocytes.

MATERIALS AND METHODS

Patient material

This study was conducted in accordance with the Declaration of Helsinki. All subjects signed written informed consent approved by the Medical Ethics Review Committee of the Amsterdam University Medical Centers (NL 64983.018.18). Peripheral blood samples were obtained from patients with SV ($n = 12$) or with stable NSV ($n = 22$) aged ≥18 years who were visiting the outpatient clinic at the Amsterdam University Medical Center (Amsterdam, The Netherlands) according to current vitiligo classification and disease activity scoring (Rodrigues et al., 2017; van Geel et al., 2019). Exclusion criterion was disease activity in the past 12 months during standard of care treatment. Similarly, we recruited healthy control subjects ($n = 20$) aged ≥18 years. The demographic characteristics of patients with vitiligo and healthy controls are represented in Table 1. PBMCs were purified from whole blood by density gradient centrifugation (LymphoPrep, Stemcell Technologies, Vancouver, Canada) and cryopreserved before analysis.

Antibodies and flow cytometry

Fluorochrome-conjugated antibodies are specified in Supplementary Table S4. Cell surface staining was performed in FACS buffer (PBS supplemented with 1% BSA and 0.05% sodium azide). Subsequently, cells were fixed in True-Nuclear Fix (BioLegend, San Diego, CA) and stained intranuclear in True-Nuclear Perm Buffer (BioLegend), according to the manufacturer's instructions. FACS acquisition was performed on a FACSCanto II B (BD Biosciences, Franklin Lakes, NJ) using BD FACSDiva software (BD Biosciences), and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Gene expression analysis

The R2 Genomics Analysis and Visualization platform (http://r2.amc.nl) was used for the analysis of gene expression profiles of patients with NSV and SV and of the healthy individuals (GSE80009) (Wang et al., 2016).

Radioligand-binding assays

Antibodies in serum samples were detected using radioligand-binding assays. Plasmids pcDNA3-TH, pcDNA3_TYR, pcDNA3-PMEL17, pcDNA3-MCHR1, and pcDNA-Melan-A (MART-1) were used according to the manufacturer's instructions in an in vitro TnT T7-coupled Reticulocyte Lysate System (Promega, Madison, WI) with [35S]-methionine to produce radiolabeled full-length TYR, TRP1, TRP2, PMEL, TYR hydroxylase, MART-1, and MCHR1, respectively. Next, radiolabeled antigens were used in radioligand-binding assays with patient sera ($n = 34$) and healthy control sera ($n = 30$) at a 1:100 dilution. The antibody index is calculated as the count per minute immunoprecipitated by tested serum divided by the mean count per minute immunoprecipitated by the group of healthy control sera (Kemp et al., 2002). Each serum was tested in at least three independent experiments. The mean antibody index was calculated from these values. Patient sera with an antibody index above the upper limit of normal (mean antibody index + 3 × SD of the healthy control individuals) were regarded as positive for antibody reactivity.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Comparisons were made with ANOVA analysis, Student's *t*-test, or Mann–Whitney test. Tukey's multiple comparisons corrections were applied for ANOVA analysis. *P*-values < 0.05 were considered statistically significant: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Data availability statement

No publicly available datasets were generated during this study. Dataset GSE80009 was used for gene expression analysis.

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Original Draft Preparation: MW, RML; Writing - Review and Editing: MW, NFP, NOPVU, VSN, SC, EHK, MWB, RML

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.05.022.

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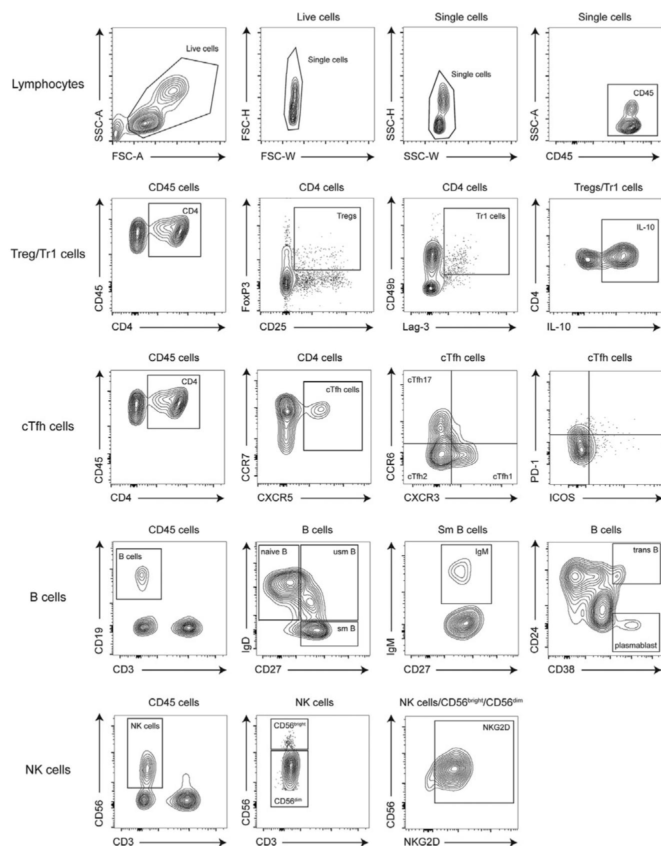
SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Gating strategy for lymphocyte subpopulations. Tregs, cTfh cells, B cells, and NK cells were analyzed in four separate flow cytometry panels. In each panel, lymphocytes were selected as CD45$^+$ cells (upper row). Tregs were considered to be CD25$^+$ FoxP3$^+$ cells from CD4$^+$ cells, and Tr1 were gated as CD49b$^+$ Lag-3$^+$ cells from CD4$^+$ cells (second row). In both Tregs and Tr1, IL-10–producing cells were studied. cTfh cells were gated as CXCR5$^+$ cells from CD4$^+$ cells (third row). On the basis of the expression of CCR6 and CXCR3, we identified cTfh1 cells (CCR6$^-$ CXCR3$^+$), cTfh2 cells (CCR6$^-$ CXCR3$^-$), and cTfh17 cells (CCR6$^+$ CXCR3$^-$). In all cTfh subsets, we studied the activation status by the markers PD-1 and ICOS. B cells were identified as CD19$^+$ CD3$^-$ cells from CD45$^+$ cells (fourth row). B cells were then classified into naive B cells (IgD$^+$ CD27$^-$), usm B cells (IgD$^+$ CD27$^+$), and sm B cells (IgD$^-$ CD27$^+$). Within the switched memory B-cell population, we analyzed the immunoglobulin subtype by staining for IgM. From B cells, we could also identify trans B cells (CD24$^+$ CD38$^+$) and plasmablasts (CD24$^-$ CD38$^+$). Finally, NK cells were considered to be CD3$^-$ CD56$^+$ cells (bottom row). Within the NK cell population, we separated CD56^{bright} and CD56^{dim} NK cells. Finally, we analyzed the expression of NKG2D on these cells. cTfh, circulating T follicular helper; FSC-A, forward scatter area; FSC-H, forward scatter height; FSC-W; forward scatter width; sm, switched memory; SSC-A, side scatter area; SSC-H, side scatter height; SSC-W, side scatter width; Tr1, type1 regulatory T cell; trans, transitional; Treg, regulatory T cell, usm, unswitched memory.

Supplementary Table S1. Differentially Expressed Genes between CD25^{high} Tregs and Conventional CD25^{-} CD4^{+} T Cells

Gene	Upregulated/Downregulated on CD25^{high} Tregs
FOXP3	Upregulated
IKZF2	Upregulated
IL2RA	Upregulated
CTLA4	Upregulated
TIGIT	Upregulated
TNFRSF18 (GITR)	Upregulated
TNFRSF4 (OX40)	Upregulated
LAG3	Upregulated
HAVCR2 (TIM-3)	Upregulated
LRRC32 (GARP)	Upregulated
ICOS	Upregulated
IL10	Upregulated
EBI3 (IL35B)	Upregulated
IL1RL1 (ST2)	Upregulated
BATF	Upregulated
LAYN	Upregulated
CSF2RB	Upregulated
TRIB1	Upregulated
ENTPD1 (CD39)	Upregulated
UTS2	Upregulated
RTKN2	Upregulated
IL7R	Downregulated
ENC1	Downregulated
NKG7	Downregulated
CD40LG	Downregulated

Abbreviations: Treg, regulatory T cell.

Supplementary Table S2. Antibody Indexes for Sera from Antibody-Positive Patients with NSV

Patient	TYR	TRP1	TRP2	PMEL	TH	MART-1	MCHR1
Patient 19	1.1	1.02	0.69	1.06	3.46	0.98	0.99
Patient 21	5.89	3.91	4.09	0.92	0.92	1.10	1.09
Patient 23	3.95	2.85	2.82	1.15	1.09	0.99	0.94
Patient 24	1.02	1.10	0.88	2.94	1.06	1.00	0.91
Patient 29	7.88	5.71	5.48	0.98	1.02	0.88	1.02
Patient 30	0.83	0.85	1.09	1.01	4.29	1.07	0.94
Patient 33	1.06	1.16	0.96	5.12	0.86	0.91	0.98
Patient 40	1.04	0.91	0.93	3.74	1.14	1.05	1.11

Abbreviations: TH, tyrosinase hydroxylase; TYR, tyrosinase.

Supplementary Table S3. Patient Characteristics

	Antibody-Positive NSV			Antibody-Negative NSV		
Characteristics	n	%	IQR/SD	n	%	IQR/SD
Total	8			14		
Age, y						
<25				1	7	
25–50	6	75		8	57	
>50	2	25		5	36	
Mean	46		(29–63)	42		(30–54)
Gender						
Male	2	25		9	64	
Female	6	75		5	36	
Skin type[1]						
Type 1	0			1	7	
Type 2	5	62.5		7	50	
Type 3	3	37.5		5	36	
Type 4	0			0		
Type 5	0			1	7	
Type 6	0			0		
Comorbidities	1	12.5		5	36	
Vitiligo age of onset (y), mean	32		±13.9	34		±19.1
Disease duration (y), median	7		(4–11.3)	8		(4.5–20)
% Affected body surface area median	1		(1–4)	1.5		(0.5–4.6)

Abbreviations: IQR, interquartile range; NSV, nonsegmental vitiligo.
[1]Skin type according to the Fitzpatrick skin scale.

Supplementary Table S4. Used Antibodies

Marker	Clone	Fluorochrome	Company	Catalog No.
CD45	2D1	BV510	BioLegend	368526
CD3	SK7	FITC	BioLegend	344804
CD56	HCD56	BV421	BioLegend	318327
CD16	3G8	PE-Cy7	BioLegend	302015
Granzyme B	QA16A02	APC/Fire 750	BioLegend	372210
CD94	DX22	PE	BioLegend	305506
NKG2D	1D11	APC	BioLegend	320808
NKp44	P44-8	PerCP-Cy5.5	BioLegend	325113
CD27	O323	PE-Cy7	BioLegend	302838
IgD	IA6-2	PE	BioLegend	348204
CD19	HIB19	APC	BioLegend	302212
CD24	ML5	PerCP-Cy5.5	BioLegend	311116
CD38	HIT2	BV421	BioLegend	303526
IgM	MHM-88	APC/Fire 750	BioLegend	314546
CXCR5	J252LD4	PE	BioLegend	356904
CXCR3	G025H7	PerCP	BioLegend	353740
CCR7	G043H7	BV421	BioLegend	353208
CCR6	G034E3	APC	BioLegend	353416
ICOS	C398.4A	APC/Fire 750	BioLegend	313536
CD49b	P1E6-C5	APC	BioLegend	359310
CD25	BC96	APC/Fire 750	BioLegend	302642
CD4	SK3	FITC	BioLegend	344604
Lag-3	11C3C65	PerCP	BioLegend	369312
CD127	A019D5	PE	BioLegend	351304
IL-10	JES3-9D7	PE-Cy7	BioLegend	501420
FOXP3	206D	BV421	BioLegend	320124
PD-1	MIH4	PE-Cy7	eBioscience	25-9969-42

Abbreviations: APC, allophycocyanin; No., number.