CHAPTER VIII

DETERMINATION OF $CD4^+/CD8^+$ RATIO AND EVALUATION OF $CD4^+CD25^{hi}FOXP3^+$ T-REGULATORY CELLS (Tregs) AND THEIR RELATION TO EFFECTOR T-CELLS (Teffs) IN GENERALIZED VITILIGO PATIENTS AND CONTROLS

8.1 INTRODUCTION

Vitiligo is an acquired depigmentation disorder characterized by loss of melanocytes from the epidermis. This condition affects approximately 0.5–1% of the world population (Taieb *et al.,* 2007). The exact etiology of vitiligo remains obscure, but autoimmunity has been strongly implicated in the development of disease, especially in generalized vitiligo (GV), because approximately 30% of vitiligo patients are affected with at least one additional autoimmune disorder (Alkhateeb *et al.,* 2003).
In addition, circulating autoantibodies (Cui and Bystryn, 1995; Kemp *et al.,* 1997), autoreactive $CD8^+$ cytotoxic T-cells and macrophages (Ogg *et al.,* 1998; Lang *et al.,* 2001; Le Gal *et al.,* 2001; Mandelcorn-Monson *et al.,* 2003; Wijngaard *et al.,* 2000) that recognize pigment cell antigens have been detected in the sera of a significant proportion of vitiligo patients compared with healthy individuals. In particular, active cases of vitiligo were demonstrated to have higher levels of autoantibodies and cytotoxic T-cells (Le Poole and Luiten, 2008). The high frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of vitiligo patients, peri-lesional T-cell infiltration and melanocyte loss *in situ* suggest the important role of cellular autoimmunity in the pathogenesis of this disease. In most vitiligo patients the balance of cytotoxic/suppressor and helper/inducer T-cells in peripheral blood is disturbed which might lead to predominance of distinct T-cell subtypes. Moreover, in progressive disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells and $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes (Wankowicz-Kalinska *et al.,* 2003). Normal values of $CD4^+$ and $CD8^+$ cells are approximately 30-60% and 10-30% respectively in healthy individuals depending on age ($CD4^+/CD8^+$ ratio: 0.9 to 3.7 in adults) (Reniz *et al.,* 1987).

Cytotoxic T-cells require $CD4^+$T-cell help to be activated against melanocytes (Steitz *et al.,* 2005). Involved helper and cytotoxic T-cells from progressing vitiligo margins generate predominantly type 1 cytokines, namely, IFN-γ and TNF-α suggesting vitiligo is a Th1-mediated disease (Le Poole *et al.,* 2004). The underlying causes for autoimmune diseases are not yet fully elucidated but autoimmunity may result from unchecked control by regulatory T-cells (Tregs) (Levings *et al.,* 2001).

A number of T-cell subsets with immunoregulatory activities have been described, and their roles in certain animal autoimmune disease models have been shown and are likely to be of relevance in human immune-mediated diseases (Maloy *et al.,* 2001). Regulatory T cells are critical for the maintenance of immune cell homeostasis. Specifically, Treg cells maintain order in the immune system by enforcing a dominant negative regulation on other immune cells. Accumulating data indicate that a deficiency or dysfunction of Tregs is associated with impaired immune homeostasis and the development of autoimmune diseases. $CD4^+CD25^+FoxP3^+$ regulatory T cells are important in maintaining self-tolerance and regulating immune responses in both physiological and pathological conditions (Sakaguchi, 2004). These naturally occurring T cells can control actively and dominantly the activation and function of autoreactive T cells that have escaped from the thymus and can prevent development of the autoimmune disease.

$CD4^+CD25^+FoxP3^+$ Tcell subset constitutively expresses high levels of CD25, the IL-2R α-chain, in addition to CD4 (Takahashi *et al.,* 1998). This population constitutes about 4-10% of peripheral $CD4^+$ cells and shows remarkable suppressive capacity both *in vitro* and *in vivo* (Suri-Payer *et al.,* 1998; Itoh *et al.,* 1999; Sasaki *et al.,* 2004). Since CD25 is also expressed in activated T-helper cells, FoxP3 was found to be a more specific marker for Tregs. FoxP3 is the acronym given to the forkhead winged-helix transcription factor "forkhead box P3" which serves as the dedicated mediator of the genetic program governing Tregs development and function, therefore it is regarded as a reliable and specific marker for Tregs (Hori *et al.,* 2003; Yagi *et al.,* 2004). Other markers expressed by Treg include GITR and CTLA-4 yet only FoxP3 expression is relatively unique to regulatory T cells (De Boer *et al.,* 2007). The transcription factor 'FoxP3' affects the expression of many genes (Zheng and Rudensky, 2007), and mutations in FoxP3 can cause severe autoimmune disorders as in immune dysregulation, polyendocrinopathy, Enteropathy X-linked (IPEX) syndrome (human) and scurfy mice, supporting the importance of Treg to keep autoreactive T cells in check (Lahl *et al.,* 2007).

$CD4^+CD25^{hi}FoxP3^+$ Tregs are further subdivided into natural, thymically derived Tregs and adaptive Tregs. Inhibition of effector cells proceeds via cell-cell contact by the former subset and via the secretion of inhibitory cytokines, mainly TGF-β by the

latter. After T-cell receptor (TCR)-mediated stimulation, $CD4^+CD25^{hi}FoxP3^+$ Tregs suppress the activation and proliferation of other $CD4^+$ and $CD8^+$ T-cells in an antigen non-specific manner (Thornton and Shevach, 2000; Thornton *et al.*, 2006).

Recently, the level of $CD4^+CD25^{hi}FoxP3^+$ Tregs and their relation to effector T-cells (Teffs) have been evaluated in generalized vitiligo demonstrating the elevation of Tregs and Teffs (Abdallah and Saad, 2009). In contrast, another study by Lili *et al.* (2012) indicated that reduced numbers and impaired function of natural Tregs fail to control the widespread activation of $CD8^+$ CTLs, which leads to the destruction of melanocytes in GV. Klarquist *et al.* (2010) showed a defect in Treg cell homing to the skin, based on the finding of drastically reduced Treg numbers in vitiligo skin without any systemic drop in their abundance or activity. However, Ben Ahmed *et al.*, identified increased number of Tregs in perilesional skin despite a functional defect of circulating Tregs in progressive vitiligo (Ben Ahmed *et al.*, 2011). Thus whether the prevalence and/or function of Tregs are truly impaired in GV patients is still controversial.

The aim of the present study was to evaluate $CD4^+/CD8^+$ ratio and $CD4^+CD25^+FoxP3^+$ Tregs and their relation to (Teffs) in patients with generalized vitiligo and compare them to unaffected controls.

8.2 MATERIALS AND METHODS

8.2.1 Patients and controls

The study group included 82 generalized vitiligo patients (including acrofacial vitiligo and vitiligo universalis) comprised of 39 males and 43 females who referred to S.S.G. Hospital, Vadodara, India (Table 1). The patients were divided into two groups based on whether the existing lesions were spreading and/ or new lesions had appeared within the previous 6 months: an affirmative answer to one or both of those questions led to inclusion of the patient in the progressive GV group, whereas patients with no increase in lesion size or number were included the stable GV group. Fifty-six patients were classified with progressive GV, whereas twenty-nine patients were included in the stable GV group. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and patients had no other associated

autoimmune diseases. A total of 50 sex-matched unaffected individuals (24 males and 26 females) were included as controls in the study (Table 1). Demographic data for patients and controls are shown in Table 1. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease.

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained in writing from all subjects before performing the studies.

Table 1. Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 82)	(n = 50)
Average age (mean age ± SD)	34.28 ± 18.95 yrs	30.41 ± 11.93 yrs
Sex: Male	39 (47.56%)	24 (48.00%)
Female	43 (52.44%)	26 (52.00%)
Age of onset (mean age ± SD)	26.69 ± 19.01 yrs	NA
Duration of disease (mean ± SD)	7.35 ± 8.51 yrs	NA
Active vitiligo	56 (68.29%)	NA
Stable vitiligo	29 (31.71%)	NA
Family history	11 (13.41%)	NA

8.2.1.1 Determination of $CD4^+/CD8^+$ ratio in vitiligo patients and controls of Gujarat

Flow cytometry

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD Biosciences, San Jose, CA). The blood samples were processed immediately within 2 h of collection, for determining the counts of $CD4^+$, $CD8^+$ cells and their ratios by two colour immunophenotyping on FACS AriaIII system (BD Biosciences, San Jose, CA), using fluorochrome labeled monoclonal antibodies to $CD4^+$ and $CD8^+$ T-cells, strictly following manufacturer's instructions (BD Simultest CD4/CD8 Reagent). The same batches of antibodies were used for testing the samples of subjects of all the groups. 100 µl of whole anticoagulated blood was lysed using 1 mL (1X) FACS Lysing Solution (BD Biosciences, San Jose, CA) followed by washing with phosphate buffer saline (PBS). After that, the cells were stained with combinations of the following antibodies (20 µl each): anti-CD4-FITC, anti-CD8-PE. The tubes were then incubated in the dark for 30 minutes followed by washing with PBS containing 0.1% sodium azide, fixed in PBS containing 1% paraformaldehyde, and acquired the same day on a FACS AriaIII system (BD Biosciences, San Jose, CA) followed by analysis using FlowJo software version 7.6.5 (Tree Star, San Carlos, CA). A doublet discrimination strategy was performed for the analysis of $CD4^+$ and $CD8^+$ T-cells (Figure 1).

8.2.1.2 Evaluation of $CD4^+CD25^{hi}FoxP3^+$ T-regulatory cells (Tregs) and their relation to effector T-cells (Teffs) in vitiligo patients and controls of Gujarat

Flow cytometry

Five ml. venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD Biosciences, San Jose, CA). All blood samples were processed immediately within 2 h of collection. Peripheral blood mononuclear cells (PBMCs) were purified on Ficoll gradients (GE Healthcare, Bjo¨rkgatan, Sweden). For the detection of natural Treg cells, PBMCs were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4, allophycocyanin (APC)-conjugated anti-CD25, and phycoerythrin (PE)-conjugated anti-FoxP3 (BD Biosciences, San Jose,

CA), according to the manufacturer's protocol. Initially, surface staining of CD4 and CD25 was done with 20 µl of respective antibodies followed by incubation of 30 min. at room temperature. For intracellular staining of FoxP3-PE (BD Biosciences, San Jose, CA): cells were fixed and permeabilized with the use of Human FoxP3 Buffer Set (BD Biosciences, San Jose, CA) according to the manufacturer's instructions with certain modifications, in brief: After washing, the cell pellet was resuspended in 0.5 ml of freshly prepared 1X fixation/ permeabilization working solution and incubated for 30 minutes at room temperature in the dark.

After staining, cells were washed twice in FACS staining buffer [phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide], fixed in PBS containing 1% paraformaldehyde, and acquired the same day on a FACS AriaIII system (BD Biosciences, San Jose, CA) followed by analysis using FlowJo software version 7.6.5 (Tree Star, San Carlos, CA). A doublet discrimination strategy was performed for the analysis of Treg cells (Figure 2).

$CD25^{hi}FoxP3^{+}$ natural Treg cells were identified within gated CD4+ T cells. To discriminate between $CD25^{hi}$ Treg and $CD25^{low}$ activated effector T-cells, we used CD25 expression on $CD8^{+}$ cells as an internal control. $CD8^{+}$ cells only express intermediate levels of CD25 ($CD25^{low}$), whereas $CD4^{+}$ T-cells express CD25 with high ($CD25^{high}$) or intermediate ($CD25^{low}$) intensities. Only $CD4^{+}$ cells expressing CD25 with higher intensities than the $CD8^{+}$ cells were included in the gate for $CD25^{high}$ cells. The gate for $CD25^{low}$ cells was set to include cells expressing CD25 at levels above those of the isotype control and unstained cells but at lower expression levels than the $CD25^{high}$ cells (Figure 5).

8.2.2 Statistical analysis

Data analysis was performed using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Quantitative data were expressed as the mean ± SEM. Statistical significance was determined by unpaired Student's t-test. *p*-values less than 0.05 were considered as statistically significant.

8.3 RESULTS

8.3.1 Determination of $CD4^+/CD8^+$ ratio in vitiligo patients and controls from Gujarat

$CD4^+$ and $CD8^+$ T-cells were identified in vitiligo patients and controls by FACS analysis as shown in Figure 1. The mean $CD4^+$ and $CD8^+$ T-cell counts in vitiligo patients and controls are given in Table 2. The $CD8^+$ cell counts were significantly higher in vitiligo patients as compared to controls (p=0.003); however, $CD4^+$ cell counts did not show significant difference between patients and controls (p=0.328) (Figure 2A). As the mean cell count of $CD8^+$ T-cell significantly increased, $CD4^+/CD8^+$ ratio decreased significantly in patients as compared to controls (Figure 2B). Analysis based on the stage of progression of vitiligo suggested increased $CD8^+$ T-cell count in patients with active vitiligo as compared to stable vitiligo (p=0.001) (Figure 3B) (Table 3). However, the mean $CD4^+$ T-cell count did not differ between active and stable vitiligo cases (p=0.579) (Figure 3A). The $CD4^+/CD8^+$ ratio in active vitiligo cases significantly lowered as compared to stable vitiligo (p=0.002) suggesting the important role of $CD8^+$ T-cell in progression of the disease (Figure 3C). Moreover, the $CD4^+$ and $CD8^+$ T-cell counts did not differ between male and female patients and hence the $CD4^+/CD8^+$ ratio did not differ (Figure 4A, B & C) suggesting there is no effect of gender biasness in $CD4^+$ and $CD8^+$ T-cell counts in patients.

When $CD4^+/CD8^+$ ratio was monitored in different age of onset groups of patients, patients with the age group 1-20 yrs did not show significant difference as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.267, p=0.318 and p=0.497 respectively) (Table 4).

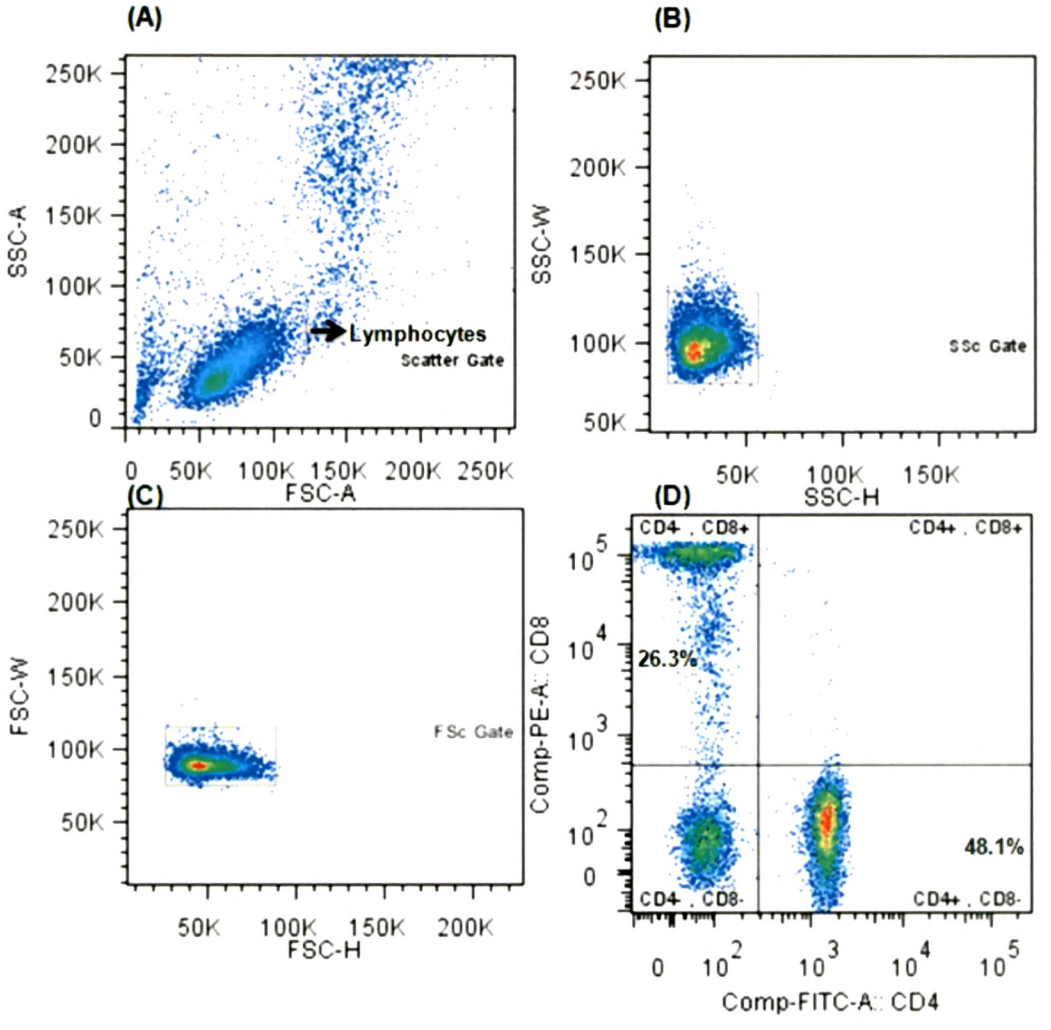


Figure 1. Human $CD4^+$ and $CD8^+$ T-cell subsets identified by FACS analysis (representative FACS plots for $CD4^+$ and $CD8^+$ T-cell):

(A) Lymphocte gating is shown in a scatter gate.

(B) & **(C)** show SSC and FSC plots for doublet discrimination of the lymphocyte population.

(D) $CD4^+CD8^-$, $CD4^-CD8^+$ cells were identified as shown.

Table 2. Counts of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ /$CD8^+$ ratio in vitiligo patients and controls.

T Cells	Vitiligo Patients (n = 82)		Controls (n = 50)		*p* Value
	Mean ± SEM (no. of cells)	Range	Mean ± SEM (no. of cells)	Range	
$CD4^+$	2984 ± 192.5	706-7482	2603 ± 377.4	602-6911	0.328
$CD8^+$	4080 ± 275.9	458-10967	2588 ± 370.2	678-5478	0.003
$CD4^+/CD8^+$	0.8326 ± 0.0502	0.3162-1.900	1.076 ± 0.0768	0.6183-1.904	0.001

n= number of patients and controls, SEM: standard error mean.

Table 3. Counts of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ /$CD8^+$ ratio in patients with active and stable vitiligo.

T Cells	Active Vitiligo (n = 56)		Stable Vitiligo (n = 29)		*p* Value
	Mean ± SEM (no. of cells)	Range	Mean ± SEM (no. of cells)	Range	
$CD4^+$	2874 ± 224.7	756-4711	3090 ± 312.7	705-7482	0.579
$CD8^+$	4793 ± 449.6	458-10967	3395 ± 269.6	570-5263	0.001
$CD4^+/CD8^+$	0.6790 ± 0.0669	0.3162-1.900	0.98 ± 0.0625	0.5711-1.666	0.002

n= number of patients and controls, SEM: standard error mean.

Table 4. Counts of $CD4^+$ and $CD8^+$ T cells and $CD4^+$ /$CD8^+$ ratio with respect to age of onset of vitiligo.

Age of Onset (yrs)	n	Subsets	Mean ± SEM	Range	95% CI	p Value
1-20	40	$CD4^+$	3033 ± 248.8	756 - 5733	2520 - 3545	-
		$CD8^+$	4042 ± 298.4	458 - 6149	3423 - 4661	-
		$CD4^+$ /$CD8^+$	0.8501 ± 0.07488	0.4789 - 1.900	0.6948 - 1.005	-
21-40	20	$CD4^+$	2958 ± 365.8	706 - 4819	2167 - 3748	0.863[a]
		$CD8^+$	4677 ± 784.2	1056 - 10967	2969 - 6386	0.375[b]
		$CD4^+$ /$CD8^+$	0.7229 ± 0.06943	0.3805 - 1.238	0.5717 – 0.8742	0.267[c]
41-60	16	$CD4^+$	2952 ± 653	745 - 7482	1446 - 4458	0.888[a]
		$CD8^+$	3314 ± 610.5	570 - 5763	1906 - 4722	0.240[b]
		$CD4^+$ /$CD8^+$	0.9992 ± 0.1370	0.4407 – 1.666	0.6833 – 1.315	0.318[c]
61-80	6	$CD4^+$	2831 ± 744.4	1414 - 4323	461.3 - 5200	0.773[a]
		$CD8^+$	4077 ± 332.4	3446 - 4805	3019 - 5135	0.963[b]
		$CD4^+$ /$CD8^+$	0.7132 ± 0.2077	0.3162 – 1.254	0.05212 – 1.374	0.497[c]

[a]Age of onset group 1-20 vs group 21-40,

[b]Age of onset group 1-20 vs group 41-60,

[c]Age of onset group 1-20 vs group 61-80,

n= number of patients, SEM: standard error mean, CI=Confidence Interval.

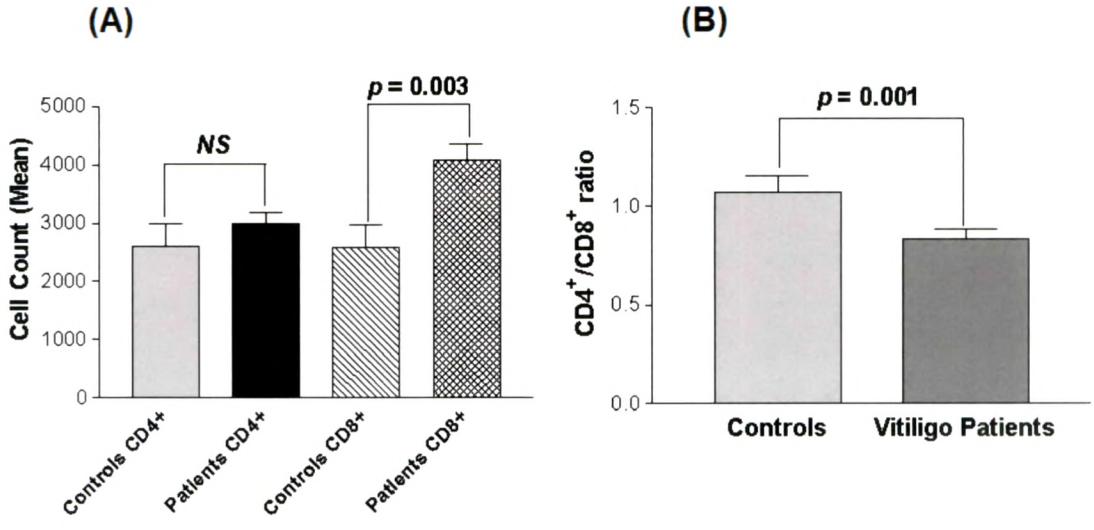


Figure 2. $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+/CD8^+$ ratio in vitiligo patients and controls:

(A) $CD4^+$ and $CD8^+$ T-cell counts in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM for $CD4^+$ controls vs patients (2603 ± 377.4 vs 2984 ± 192.5; p=0.328); $CD8^+$ controls vs patients (2588 ± 370.2 vs 4080 ± 275.9; p=0.003); (NS=non-significant).

(B) $CD4^+/CD8^+$ T-cell ratio in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (1.076 ± 0.0768 vs 0.8326 ± 0.0502; p=0.001).

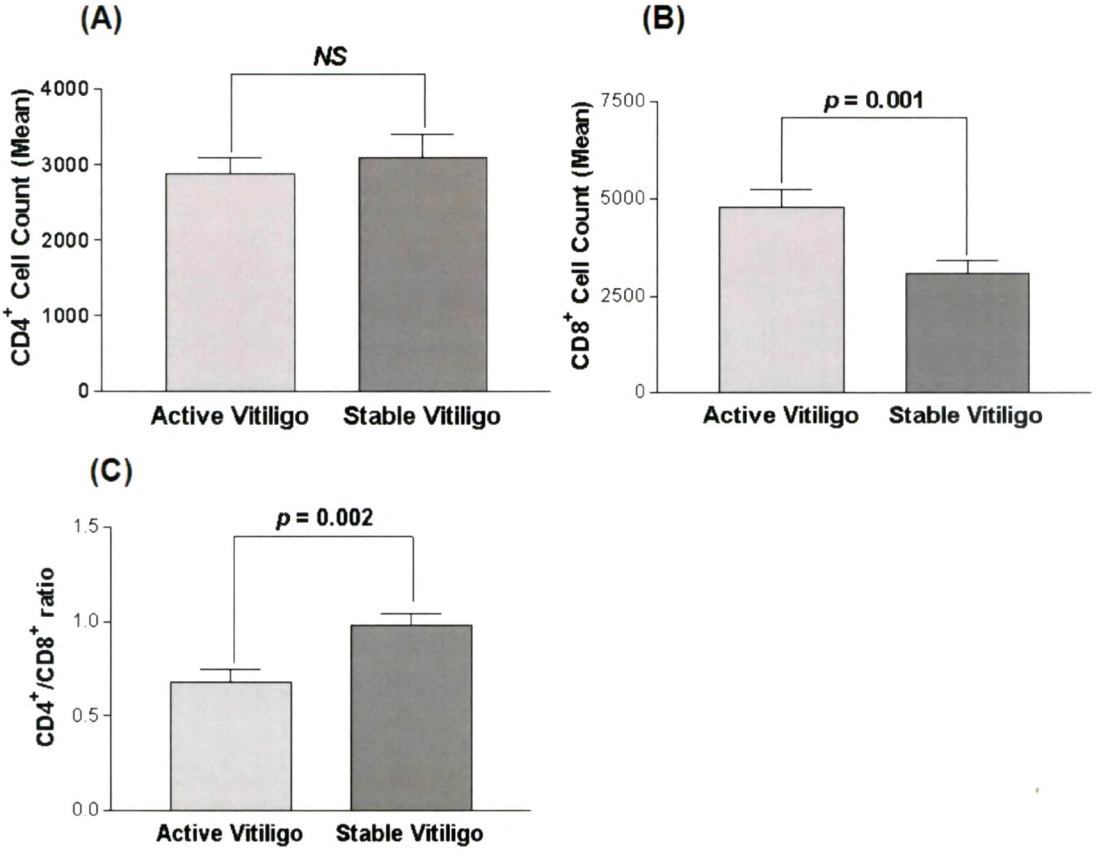


Figure 3. $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+/CD8^+$ ratio in patients with active and stable vitiligo:

(A) $CD4^+$ T-cell counts in active (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: Active vs Stable (2874 ± 224.7 vs 3090 ± 312.7; p=0.579); (NS=non significant).

(B) $CD8^+$ T-cell counts in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (4793 ± 449.6 vs 3395 ± 269.6; p=0.001).

(C) $CD4^+/CD8^+$ T-cell ratio in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (0.6790 ± 0.0669 vs 0.98 ± 0.0625; p=0.002).

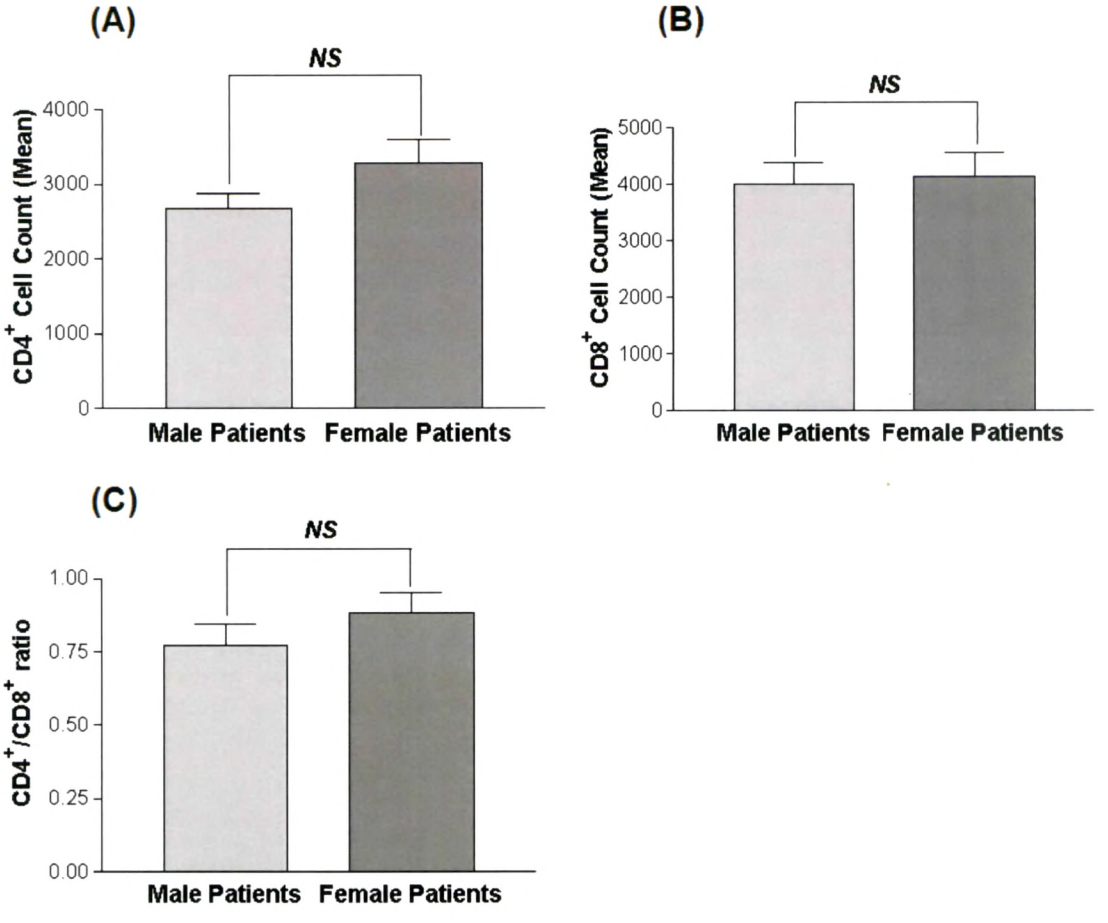


Figure 4. $CD4^+$ and $CD8^+$ T-cell counts and $CD4^+/CD8^+$ ratio in male and female patients with vitiligo:

(A) $CD4^+$ T-cell counts in male (n = 39) and female vitiligo patients (n = 43). Values are given as mean ± SEM: male vs female (2674 ± 210.9 vs 3282 ± 311.9; p=0.116); (NS=non significant).

(B) $CD8^+$ T-cell counts in male (n = 39) and female vitiligo patients (n = 43). Values are given as mean ± SEM: male vs female (4008 ± 372.2 vs 4143 ± 409.1; p=0.811); (NS=non significant).

(C) $CD4^+/CD8^+$ T-cell ratio in male (n = 39) and female vitiligo patients (n = 43). Values are given as mean ± SEM: active vs stable (0.774 ± 0.07217 vs 0.8844 ± 0.06955; p=0.277); (NS=non significant).

8.3.2 Evaluation of $CD4^+CD25^{hi}FoxP3^+$ T-regulatory cells (Tregs) and their relation to effector T-cells (Teffs) in vitiligo patients and controls from Gujarat

8.3.2.1 Defining the human Treg cell population

Using the strict gating parameters reported previously (Baecher-Allan *et al.,* 2001), Tregs were identified as $CD4^+$ cells with higher CD25 expression than that of the $CD4^-$ population (Figure 5E). The frequency of Treg was calculated as the percentage of $CD4^+CD25^{hi}$ cells in the $CD4^+$ population and was in the range of 4.192-12.92% of $CD4^+$ cells in PBMC of healthy age-matched controls. The $CD25^{hi}$ population was further analyzed by the expression of FoxP3 (Figure 5F).

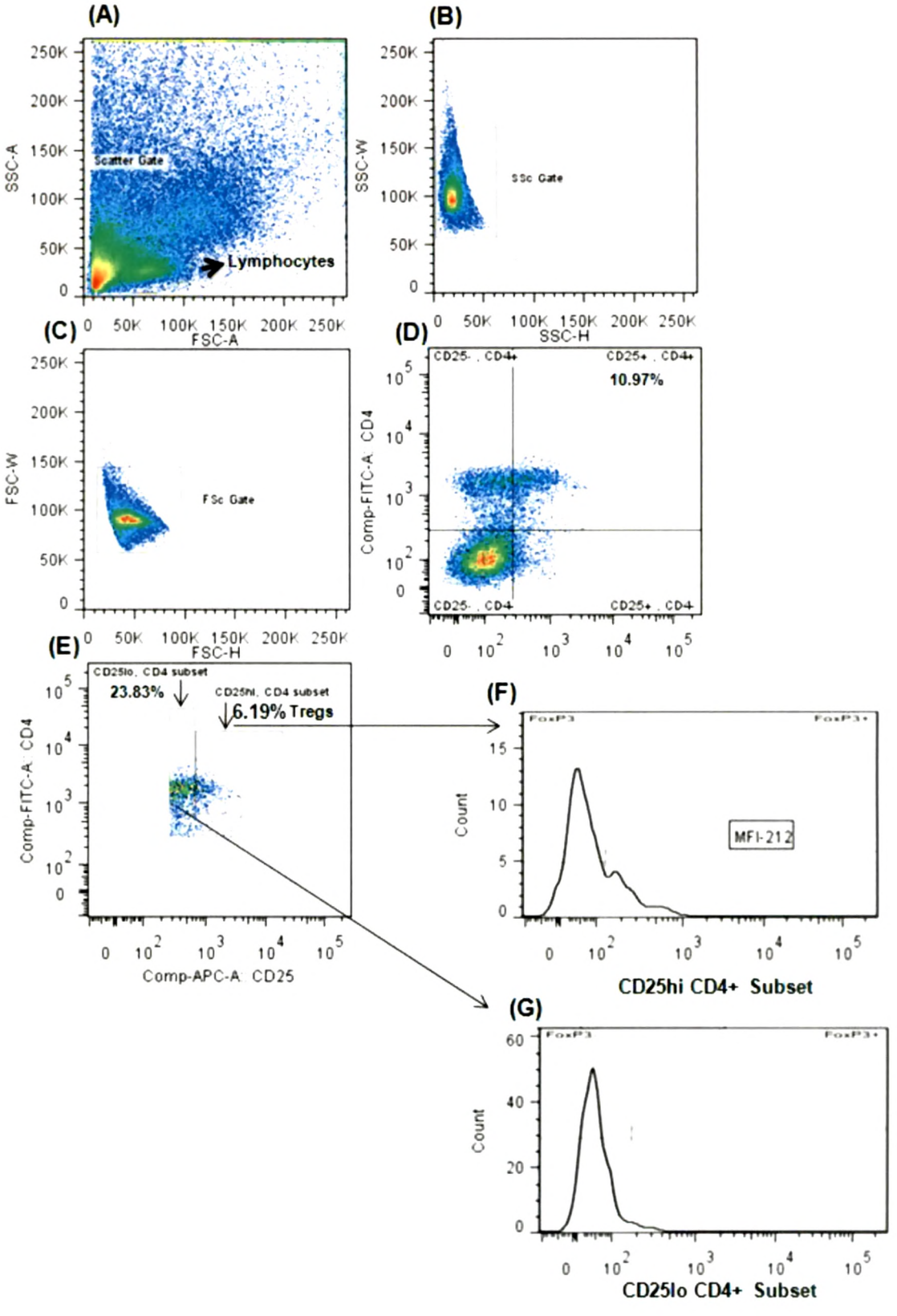

(A)
SSC-A
FSC-A
Scatter Gate
Lymphocytes
(B)
SSC-W
SSC-H
SSc Gate
(C)
FSC-W
FSC-H
FSc Gate
(D)
Comp-FITC-A :: CD4
CD25- , CD4+
CD25+ , CD4+
10.97%
CD25- , CD4-
CD25+ , CD4-
(E)
CD25lo, CD4 subset
23.83%
CD25hi, CD4 subset
6.19% Tregs
Comp-APC-A :: CD25
(F)
FoxP3
FoxP3+
Count
MFI-212
CD25hi CD4+ Subset
(G)
FoxP3
FoxP3+
Count
CD25lo CD4+ Subset

Figure 5. Characterization of human Treg ($CD4^+CD25^{hi}FoxP3^+$) population by FACS analysis (representative FACS plots for Treg ($CD4^+CD25^{hi}FoxP3^+$) cells):
(A) Lymphocte gating is shown in a scatter gate.
(B) & **(C)** show SSC and FSC plots for doublet discrimination of the lymphocyte population.
(D) $CD4^+CD25^-$, $CD4^-CD25^+$ and $CD4^+CD25^+$ cells were identified as shown.
(E) $CD4^+$CD25hi cells are those where CD25 expression was higher than that on the $CD4^-$ population. $CD4^+$CD25lo $FoxP3^-$ (Teffs) population was also identified.
(F) FoxP3 expression within $CD4^+CD25^{hi}$ and $CD4^+CD25^{lo}$ populations was assessed by intracellular staining.

8.3.2.2 Decrease of $CD4^+CD25^{hi}FoxP3^+$ Treg cells in vitiligo patients

There was significant decrease in $CD4^+CD25^{hi}FoxP3^+$ Treg% in generalized vitiligo patients (6.538 ± 0.2863) (range 1.075-9.711) compared to healthy controls (8.122 ± 0.5893) (range 4.192-12.92) (p=0.009) (Figure 6A). FoxP3 expression in Tregs was significantly reduced in $CD4^+CD25^{hi}$ T-cells of patients (592.3 ± 68.03) compared to controls (867.5 ± 52.10) (p=0.024) (Figure 6B).

Analysis based on the stage of progression of vitiligo suggested that percentage of $CD4^+CD25^{hi}FoxP3^+$ Treg cells was significantly decreased in the 56 patients with active cases (6.043 ± 0.4185) compared to 29 patients with stable vitiligo (7.731 ± 0.4334) (p=0.007) (Figure 6C). However, FoxP3 expression in Tregs did not differ between cases of active and stable vitiligo (p=0.779) (Figure 6D). Moreover, gender based analysis of $CD4^+CD25^{hi}FoxP3^+$ Treg cells did not show significant difference in Treg% between male and female patients (p=0.901) (Figure 6E). Interestingly, female patients showed significant decrease in expression of FoxP3 in Tregs (532.7 ± 79.10) as compared to male patients (908.1 ± 137.9) (p=0.021) (Figure 6F).

When $CD4^+CD25^{hi}FoxP3^+$ Treg cells were monitored in different age of onset groups of patients, patients with the age group 1-20 yrs did not show significant difference in Treg% as compared to the age group 21-40 yrs (p=0.793). However, age of onset group 1-20 showed significant decreased Treg% (6.303 ± 0.4351) as compared to age groups 41-60 yrs (8.379 ± 0.5612) and 61-80 yrs (9.610 ± 1.199) (p=0.027 and p=0.010 respectively) (Figure 6G). There was no significant difference in expression

of FoxP3 in Tregs when age of onset group 1-20 yrs (p= 0.060) was compared with age groups 41-60 and 61-80 yrs (p=0.191 and p=0.214 respectively) (Figure 6H).

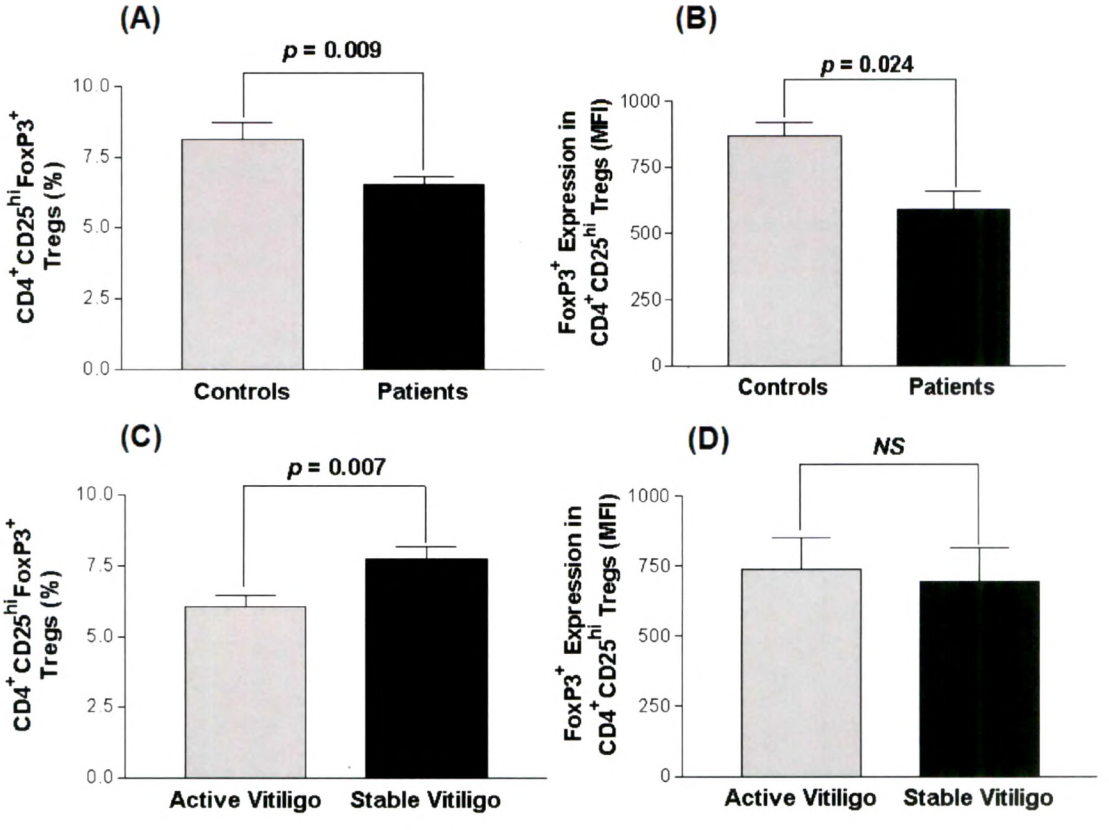


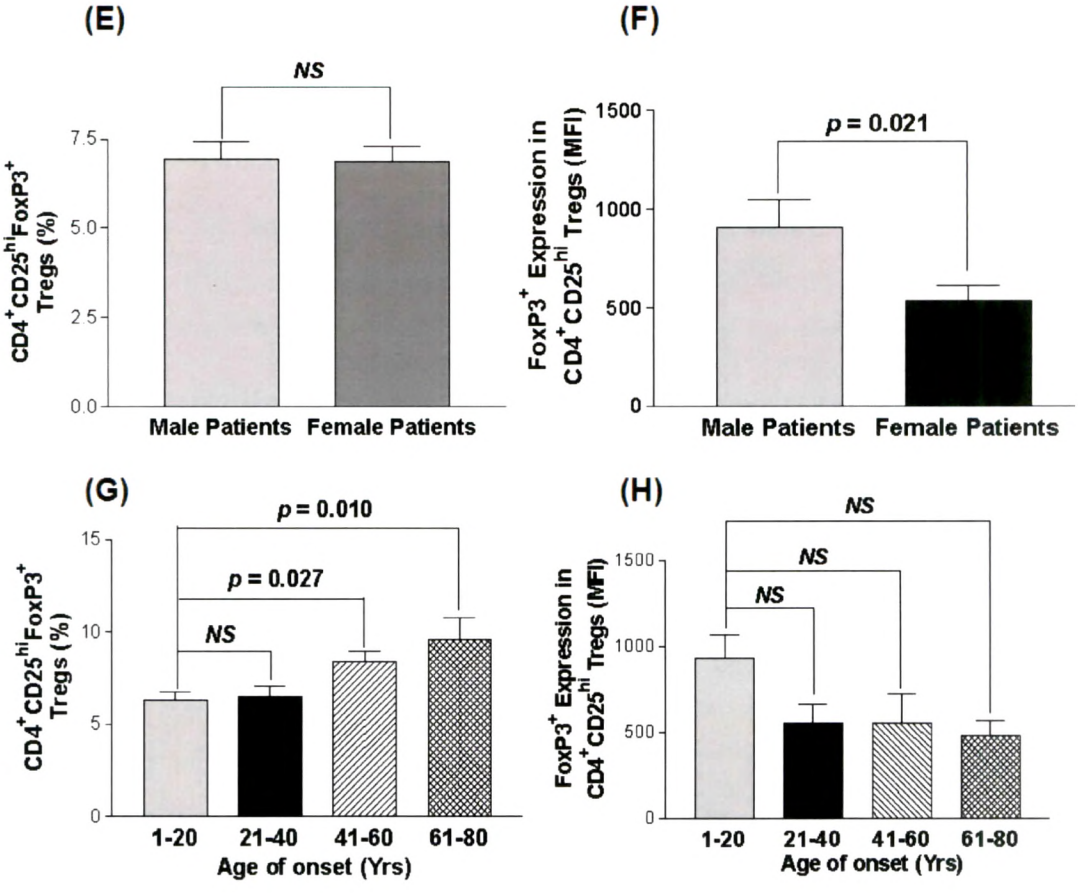
Figure 6. $CD4^+CD25^{hi}Foxp3^+$ Treg cells in vitiligo patients and controls:

(A) $CD4^+CD25^{hi}Foxp3^+$ Treg cells in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (8.122 ± 0.5893 vs 6.538 ± 0.2863; p=0.009).

(B) Foxp3 expression in $CD4^+CD25^{hi}$ Tregs in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (867.5 ± 52.10 vs 592.3 ± 68.03; p=0.024).

(C) $CD4^+CD25^{hi}FoxP3^+$ Treg cells in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (6.043 ± 0.4185 vs 7.731 ± 0.4334; p=0.007).

(D) FoxP3 expression in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (740.7 ± 112.9 vs 693.9 ± 121.1; p=0.779); (MFI=Median Fluorescence Intensity).



(E) $CD4^+CD25^{hi}FoxP3^+$ Treg cells in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (6.943 ± 0.4768 vs 6.864 ± 0.4396; p=0.903); (NS=non-significant).

(F) FoxP3 expression in $CD4^+CD25^{hi}$ Tregs in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (908.1 ± 137.9 vs 532.7 ± 79.10; p=0.021); (MFI=Median Fluorescence Intensity).

(G) $CD4^+CD25^{hi}Foxp3^+$ Treg cells in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (6.303 ± 0.4351 vs 6.495 ± 0.5905, 8.379 ± 0.5612, 9.610 ± 1.199; p=0.793, p=0.027 and p=0.010 respectively).

(H) FoxP3 expression in $CD4^+CD25^{hi}$ Tregs in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (927.8 ± 136.5 vs 552.6 ± 107.9, 554.9 ± 166.2, 476.8 ± 91.09; p=0.060, p=0.191 and p=0.214 respectively); (MFI=Median Fluorescence Intensity).

8.3.2.3 Increase of T-effector ($CD4^+CD25^{low}FoxP3^-$) cells in vitiligo patients

There was significant increase in $CD4^+CD25^{low}FoxP3^-$ responsive or effector T-cells (Teffs) in vitiligo patients (14.98 ± 0.7445) compared to controls (10.16 ± 1.031) (p=0.001) (Figure 7A). Analysis based on the stage of progression of vitiligo suggested that percentage of $CD4^+CD25^{low}FoxP3^-$ Teff cells was significantly increased in the 56 patients with active cases (13.96 ± 1.010) compared to 29 patients with stable vitiligo (11.09 ± 0.8013) (p=0.029) (Figure 7B).

Further, gender based analysis of $CD4^+CD25^{low}FoxP3^-$ Teff cells did not show significant difference in Teff% between male and female patients (p=0.996) (Figure 7C). Interestingly, when $CD4^+CD25^{low}FoxP3^-$ Teff cells were monitored in different age of onset groups of patients, patients with the age group 1-20 yrs (10.79 ± 0.6943) showed significant difference in Teff% as compared to the age groups 21-40 yrs (14.62 ± 1.275), 41-60 yrs (14.51 ± 1.891) and 61-80 yrs (15.05 ± 2.481) (p=0.006, p=0.032 and p=0.044 respectively) (Figure 7D).

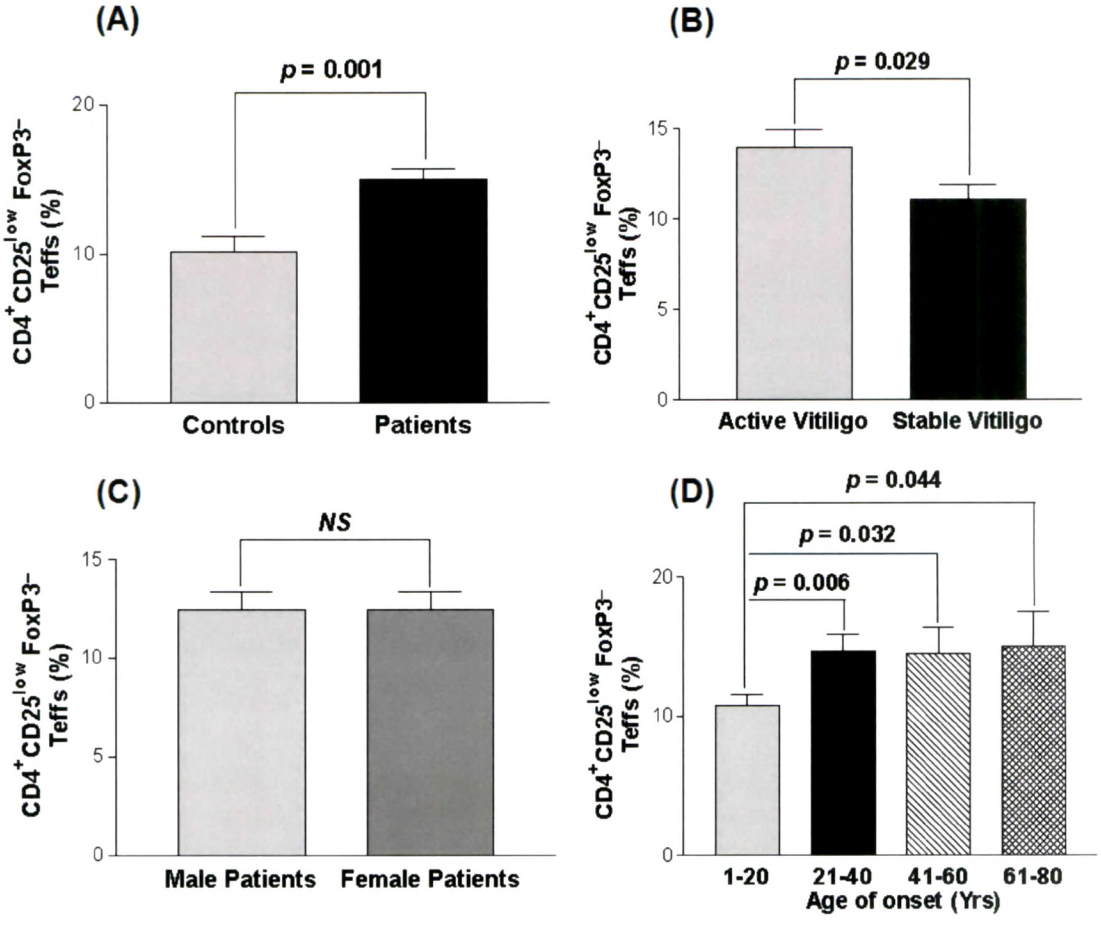


Figure 7. T-effector ($CD4^+CD25^{low}FoxP3^-$) cells in vitiligo patients and controls:

(A) $CD4^+CD25^{low}FoxP3^-$ Teff cells in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (10.16 ± 1.031 vs 14.98 ± 0.7445; p=0.001).

(B) $CD4^+CD25^{low}FoxP3^-$ Teff cells in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (13.96 ± 1.010 vs 11.09 ± 0.8013; p=0.029).

(C) $CD4^+CD25^{low}FoxP3^-$ Teff cells in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (12.45 ± 0.9285 vs 12.44 ± 0.9595; p=0.996) (NS=non-significant).

(D) $CD4^+CD25^{low}FoxP3^-$ Teff cells in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (10.79 ± 0.6943 vs 14.62 ± 1.275, 14.51 ± 1.891, 15.05 ± 2.481; p=0.006, p=0.032 and p=0.044 respectively).

8.3.2.4 Ratio of Tregs to Teffector (Tregs/Teffs) cells in vitiligo patients

The Tregs/Teffs ratio (0.5420 ± 0.02668) was significantly lower in vitiligo patients than that of unaffected controls (0.6879 ± 0.07651) (p=0.025) (Figure 8A). Analysis based on the stage of progression of vitiligo suggested that ratio of Tregs/Teffs was significantly decreased in the 56 patients with active cases (0.5124 ± 0.03684) compared to 29 patients with stable vitiligo (0.7086 ± 0.05222) (p=0.003) (Figure 8B).

Further, gender based analysis of Tregs/Teffs ratio did not show significant difference between male and female patients (p=0.559) (Figure 8C). Also, when Tregs/Teffs ratio was analyzed in different age of onset groups of patients, patients with the age group 1-20 yrs did not show significant difference as compared to the age groups 21-40, 41-60 and 61-80 yrs (p=0.741, p=0.874 and p=0.387 respectively) (Figure 8D).

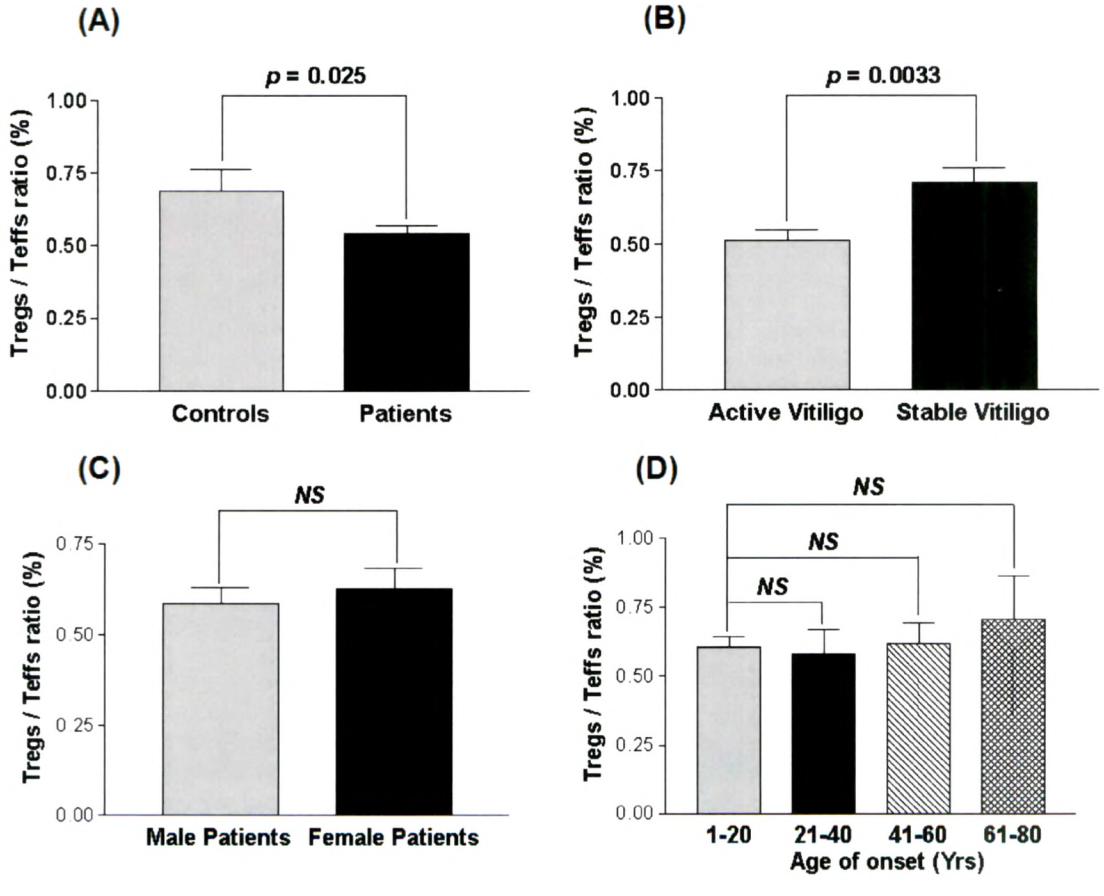


Figure 8. Ratio of Tregs to Teffector (Tregs/Teffs) cells in vitiligo patients and controls:

(A) Tregs/Teffs ratio in vitiligo patients (n = 82) and controls (n = 50). Values are given as mean ± SEM: controls vs patients (0.6879 ± 0.07651 vs 0.5420 ± 0.02668; p=0.025).

(B) Tregs/Teffs ratio in active vitiligo (n = 56) and stable vitiligo patients (n = 29). Values are given as mean ± SEM: active vs stable (0.5124 ± 0.03684 vs 0.7086 ± 0.05222; p=0.003).

(C) Tregs/Teffs ratio in male (n = 39) and female patients (n = 43) with vitiligo. Values are given as mean ± SEM: male vs female (0.5880 ± 0.04181 vs 0.6286 ± 0.05439; p=0.559); (NS=non-significant).

(D) Tregs/Teffs ratio in different age of onset groups of patients (1-20 yrs, n = 40; 21-40 yrs, n=20; 41-60 yrs, n=16, 61-80 yrs, n=6). Values are given as mean ± SEM: 1-20 yrs vs 21-40, 41-60, 61-80 yrs (0.6071 ± 0.03855 vs 0.5794 ± 0.08790, 0.6206 ± 0.07374, 0.7080 ± 0.1549; p=0.741, p=0.874 and p=0.387 respectively); (NS=non-significant).

8.4 DISCUSSION

T cells are more prevalent in vitiligo perilesional skin than in surrounding non-lesional or lesional skin. An occasional T cell remains in lesional skin but most of the infiltrate appears to migrate with the depigmenting epidermal border. Among the T cells, the infiltrate consists mainly of $CD8^+$ but also of $CD4^+$ T cells (Le Poole *et al.*, 1996). Since cytotoxic T cells are especially prevalent and colocalize with remaining melanocytes, it was postulated that these T cells are actively cytolytic towards remaining melanocytes (Wankowicz-Kalinska *et al.*, 2003). The involvement of T cells in vitiligo is further supported by the observation that depigmentation is accompanied by the expression of type 1 cytokines (Le Poole *et al.*, 2004). Moreover, studies have previously demonstrated that melanocytes are capable of phagocytosis and can process and present antigens in the context of MHC class II to $CD4^+$ proliferative and cytotoxic T cells (Le Poole *et al.*, 1993a,b).

Association of Major Histocompatibility Complex (MHC) alleles with vitiligo gains importance because of the antigen-presenting function of the MHC. Our recent study suggests a consistent increase of *A*33:01*, *B*44:03*, and *DRB1*07:01*, implicating these alleles as possible markers of vitiligo in North India and Gujarat These data apparently suggest auto reactive CD4+ T-helper cells to be restricted by HLA-

*DRB1*07:01* and the auto-reactive CD8+ cytotoxic T cells by *HLA-A*33:01*, *A*02:01*, *B*44:03*, and *B*57:01* in the Indian populations studied (Singh *et al.*, 2012). In another recent study, we identified the three most significant class II region SNPs: rs3096691 (just upstream of *NOTCH4*), rs3129859 (just upstream of HLA-DRA), and rs482044 (between *HLA-DRB1* and *HLA-DQA1*) (unpublished data) associated with generalized vitiligo suggesting an important link between vitiligo and immune system.

Currently, studies presenting the reciprocal relationship between $CD4^+CD25^+Foxp3^+$ Tregs and $CD8^+$ in GV progression are lacking. $CD8^+$ Tcell-mediated tissue damage has been demonstrated in common organ-specific autoimmune diseases, such as type I diabetes and multiple sclerosis, and a role for $CD8^+$ T cells has been postulated in the pathogenesis of GV. Previous studies have largely focused on melanocyte-specific cytotoxic T lymphocytes (CTLs) and identified their pivotal role in inducing melanocyte destruction (Ogg *et al.*, 1998, Palermo *et al.*, 2001, Lang *et al.*, 2001). Several reports have shown that increase in globally activated $CD8^+$ CTLs correlate with disease activity in various autoimmune disorders (Blanco *et al.*, 2005; Giovanni *et al.*, 2011). Moreover, in progressive disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells and $CD8^+$ T cells isolated from vitiligo skin are cytotoxic to melanocytes (Wankowicz-Kalinska *et al.*, 2003). Recently, Lili *et al.* (2012) have demonstrated significantly higher number of circulating $CD8^+$ T among $CD3^+$ T cells in progressive GV.

A decrease in $CD4^+$ T-cell population and an increase in $CD8^+$ cells have also been observed in vitiligo cases by Grimes *et al.* (1986) and Halder *et al.* (1986). The present study also showed decreased $CD4^+/CD8^+$ ratio in vitiligo patients as compared to controls, indicating the prevalence of $CD8^+$ cells in patients. In addition, the peripheral $CD8^+$ cells were prevalent in active cases of vitiligo as compared to stable cases; however, there was no effect of age of onset and gender biasness on $CD4^+/CD8^+$ ratio in patients suggesting that the decreased $CD4^+/CD8^+$ ratio mainly affects the progression of the disease.

Recently, decreased ratio of $CD4^+/CD8^+$ and increase in $CD8^+$ cells was demonstrated in vitiligo patients from India suggested important role of $CD8^+$ cells in the T cell mediated melanocyte death (Nigam *et al.*, 2011). However, the study could not

achieve stastical significance for the $CD4^+/CD8^+$ ratio in active and stable cases of vitiligo. In contrast, Pichler *et al.* (2009) reported increased $CD4^+/CD8^+$ ratio in vitiligo patients; however 40% of their patients had associated autoimmune thyroiditis which might have influenced their reults. Thus far, the immune mechanisms underlying the induction and activation of autoreactive $CD8^+$ CTLs and the loss of tolerance to auto-antigens are not clear. MART-1 and gp100 were first identified as the target antigens for T cells infiltrating melanoma tumors suggesting why the autoimmune response to melanocytes effectively eliminates melanocytes from vitiligo skin whereas T cells infiltrating melanomas fail to clear the tumor. An abundance of Tregs in tumor tissues is thought to be the root cause of failing attempts to boost anti-tumor immunity by powerful vaccines, and inclusion of FoxP3 as a target antigen in vaccines was shown to boost anti-tumor immunity (Loddenkemper *et al.*, 2009; Nair *et al.*, 2007). It has been proposed that failure to suppress an ongoing immune response to self antigens may contribute to progressive depigmentation of the skin in vitiligo patients (Das *et al.*, 2001).

Natural Treg cells play a key role in maintaining peripheral tolerance *in vivo* through the active suppression of self-reactive T cell activation and expansion (Klarquist *et al.*, 2010). Dysfunction or deficiency of Tregs has been reported in several autoimmune diseases, both systemic (Antiga *et al.*, 2010; Miyara *et al.*, 2005) and organ-specific (Chi *et al.*, 2008).

The most intriguing Tregs are those showing $CD4^+CD25^{hi}FoxP3^+$ phenotype. $CD4^+CD25^{hi}$ cells constitute 4-10% whereas $CD4^+CD25^{low}$ cells comprise 22-23% of peripheral blood lymphocytes (Sasaki *et al.*, 2004; Sugiyama *et al.*, 2005). Several other markers have also been described for Tregs, such as surface-bound latency-associated peptide/transforming growth factor β1 (LAP/TGF-β1), glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), neuropilin-1, galectin-1, and lymphocyte activation gene-3 (LAG-3). Furthermore, toll like receptors (TLRs), mainly expressed on antigen-presenting cells (APC) as a bridge linking innate and adaptive immunity, also exist on Tregs (Zhang *et al.*, 2008). These Tregs play a major role in tuning inflammatory response to infections, in guarding against autoimmunity and play a role in tumor progression (Afzali *et al.*, 2007). It has been proposed from murine

melanoma studies, that depletion of Tregs would activate antimelanoma cytotoxic T-cells that destroy melanoma and generate vitiligo as a side effect (Zhang *et al.*, 2007). Regulatory T cells are known to inhibit autoreactivity, explaining why autoreactive T cells are present in the circulation in the absence of autoimmune symptoms (Baecher-Allen and Hafler, 2006). While the mechanism of action for Tregs is still not fully understood, TGF-β and IL-10 contribute to Treg mediated immunosuppression. TGF-β is important for imposing a regulatory phenotype to the Treg subset and regulatory activity is dependent on cell-cell contact (Bala and Moudgil, 2006; Joetham *et al.*, 2007; Zhu and Paul, 2008).

The level of $CD4^+CD25^{hi}$ $FoxP3^+$ Tregs has been variably reported in different autoimmune diseases. A decrease in the proportion of circulating Tregs among other $CD4^+$ T-cells is reported in active cases of systemic lupus erythematosus. In contrast, the proportions of Tregs in primary Sjögren syndrome, inflammatory myopathy (Miyara *et al.*, 2005), type I diabetes mellitus (Lindley *et al.*, 2005) and multiple sclerosis (Viglietta *et al.*, 2004) do not significantly differ from controls values. As for autoimmune skin diseases, Tregs were normal in number in serum of psoriatic patients (Sugiyama *et al.*, 2005) and were decreased in pemphigus vulgaris (Sugiyama *et al.*, 2007).

Currently, very little is known about the function of Treg cells in GV patients. To date, a few studies have investigated Treg numbers or function in GV patients. In this study, we show that GV patients exhibit a decreased prevalence of circulating Tregs; however a recent study has demonstrated that the percentage of peripheral $CD4^+CD25^+FoxP3^+$Tregs remains unaltered in generalized vitiligo (Zhou *et al.*, 2012). Our results are in concordance with those of Lili *et al.*, 2012 indicating that reduced numbers and impaired function of natural Tregs fail to control the widespread activation of $CD8^+$CTLs, which leads to the destruction of melanocytes in GV. Nevertheless, Abdallah and Saad evaluated the level of $CD4^+CD25^{hi}FoxP3^+$ Tregs in generalized vitiligo and their relation to effector T-cells (Teffs) demonstrating the elevation of Tregs and Teffs (Abdallah and Saad, 2009). Klarquist *et al.*, revealed a defect in Treg cell homing to the skin, based on the finding of drastically reduced Treg numbers in vitiligo skin without any systemic drop in their abundance or activity (Klarquist *et al.*, 2010). However, a recent report identified increased numbers of

Tregs in perilesional skin despite a functional defect of circulating Tregs in progressive vitiligo (Ben Ahmed *et al.,* 2011). Moreover, in the present study FoxP3 expression in the $CD4^{+}CD25^{hi}$ Treg cells was found to be significantly decreased in cases of vitiligo as compared to controls suggesting that the Tregs may have functional defects which can affect their suppressive activity. Patients with an autoimmune disease such as systemic lupus erythematosus have a relative dysfunction of FoxP3 positive cells (do-Sánchez *et al.,* 2006). The dysfunction of FoxP3 may be due to mutations e.g. in the X-linked IPEX syndrome (immunodysregulation polyendocrinopathy and enteropathy X-linked) mutations were in the forkhead domain of FoxP3, indicating that the mutations may disrupt its critical DNA interactions (Bennett *et al.,* 2001). Recently, Lili *et al.* (2012) and Ben Ahmed *et al.* (2011) have demonstrated that the suppressive effects of peripheral T regulatory cells in progressive GV cases were significantly reduced, demonstrating an impairment in their ability to inhibit the proliferation and cytokine production of stimulated autologous $CD8^{+}$ T cells. Gerold *et al.,* (2011) also showed decreased sCTLA-4 levels in type-1 diabetic condition and suggested that lower sCTLA-4 expression may directly affect the suppressive capacity of Treg cells and thereby modulate disease risk. Our findings also suggested decreased levels of both sCTLA-4 and flCTLA-4 suggestive of the disturbance in the suppressive capacity of Tregs in vitiligo patients (Dwivedi *et al.,* 2011). Thus, a functional defect in Tregs might be involved in the pathogenesis of vitiligo.

Interestingly, our results suggest that female patients showed significant low expression of FoxP3 in $CD4^{+}CD25^{h}$i Treg cells as compared to male patients suggestive of the fact that females have increased susceptibility towards vitiligo as compared to males, implicating sex bias in the development of autoimmunity (Whitacre, 2001; Panchanathan and Choubey, 2012; Afshan *et al.,* 2012). However, we did not find the significant difference in Treg% between female and male patients with vitiligo.

In addition, we found decreased Treg cells in early age group of patients (1-20 yrs) as compared to the late onset groups which suggests the crucial role of Treg cells in early phase of the disease. Moreover, the Teff cells were found to be increased in vitiligo patients as compared to controls suggesting the effect of reduced number

and/dusfunction of Treg cells in patients. Also, active cases of vitiligo showed significant increase in Teff cells which further signifies the importance of Tregs in progression of the disease. Previous studies showed increased Teff cells in progressing vitligo (Abdallah and Saad, 2009; Lili *et al.,* 2012). Furthermore, the Treg/Teff cells ratio was found to be decreased in vitligo patients as compared to controls suggesting the increased Teff cells in patients. The Treg/Teff cells ratio was significantly decreased in active cases of vitligo as compared to stable cases, indicating the role of Teff cells in progression of the disease.

It is possible that high affinity T cells previously escaped clonal deletion in skin-draining lymph nodes and were inadvertently allowed to enter the circulation, emigrating to the skin to inflict damage to the melanocyte population (Palermo *et al.,* 2005). An ongoing autoimmune response may be allowed to further develop and mature in the absence of functional T regulatory cells (Tregs) (Dejaco *et al.,* 2006). These cells actively mediate suppression of the immune system generally by secreting IL-10 and TGF-β to prevent autoimmunity (Dejaco *et al.,* 2006). Our recent data suggested that IL-10 expression is decreased in vitiligo patients as compared to controls indicating that decreased levels of IL-10 may affect the supressive capacity of Tregs. The present study showing a decrease in the number of Tregs in vitiligo skin indicates that this may apply to vitiligo as well. In the absence of regulatory T cells, cytotoxic T cells with increasing affinity for their targets enter the skin and continuously proliferate and migrate towards novel target cells, causing depigmentation.

In conclusion, our study indicates that an imbalance of $CD4^+/CD8^+$ ratio and natural Tregs in frequency and function might be involved in the T-cell mediated pathogenesis of vitiligo and its progression. The reduced levels of natural Treg cell population might lead to a global expansion and widespread activation of the $CD8^+$ population, which could result in the destruction of melanocytes in vitiligo patients.

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