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Systemic analyses of immunophenotypes of peripheral T cells in non-segmental vitiligo: implication of defective natural killer T cells

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Summary

Although it is widely believed that non-segmental vitiligo (NSV) results from the autoimmune destruction of melanocytes, a clear understanding of defects in immune tolerance, which mediate this uncontrolled self-reactivity, is still lacking. In the present study, we systemically evaluated circulating regulatory T (Treg) cells, including $CD4^+CD25^+FoxP3^+$ Treg cells and invariant natural killer T (*i*NKT) cells, as well as naïve and memory $CD4^+$ and $CD8^+$ T cells and their cytokine production, in a cohort of 43 progressive NSV patients with race-, gender-, and age-matched healthy controls. We found that the general immunophenotypes of $CD4^+$ and $CD8^+$ T cells and the percentage of $CD4^+CD25^+FoxP3^+$ Tregs were comparable between NSV and healthy controls. However, percentages of peripheral *i*NKT cells were significantly decreased in NSV patients compared to that in healthy controls. Our data confirm the previous notion that the percentage of peripheral $CD4^+CD25^+FoxP3^+$ Tregs remains unaltered in NSV and suggests the involvement of defective *i*NKT cells in the pathogenesis of NSV.

Keywords

invariant natural killer T cells; non-segmental vitiligo; regulatory T cells; immunophenotypes

Introduction

Vitiligo is a chronic skin disorder, characterized by progressive skin depigmentation because of the loss of melanocytes. There are two broad types of vitiligo: segmental vitiligo (SV) and NSV, with the latter comprising several topographic and extent variants. At the Vitiligo Global Issues Consensus Conference held during the 2011 International Pigment Cell Conference, it was suggested that SV should be classified separately from all other forms of

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vitiligo (V). Although not fully satisfactory, the term NSV is currently used as an *umbrella term* for different clinical subtypes of vitiligo, which are all clearly different from SV, including acrofacial, generalized, mucosal (multifocal), and universal (Ezzedine et al., 2012). The prevalence of NSV in general population is approximately 0.5–1%; it accounts for up to 90% of total vitiligo cases (Spritz, 2010; Taieb and Picardo, 2009). The pathogenesis of NSV is still not completely understood, although many hypotheses have been proposed, including autoimmune, cytotoxic (an intrinsic defect of melanocytes), oxidative damage, and neural mechanisms. The destruction of melanocytes by autoimmune mechanism is the major hypothesis toward explaining the pathogenesis of NSV. Results from recent genome-wide association studies identified NSV susceptibility genes that are almost exclusively involved in biologic pathways related to immune regulation and immune targeting of melanocytes, further supporting the hypothesis of NSV as a primary autoimmune disease (Jin et al., 2007, 2010; Quan et al., 2010; Spritz, 2012). Recently, three additional susceptibility loci, namely thymic stromal lymphopoietin, FOXP3 (forkhead box P3), and CTLA-4 loci were found to be associated with NSV (Birlea et al., 2011). These three loci have been also clearly shown to have a critical role in T-cell differentiation and proliferation, further suggesting a relationship between immune disregulation and NSV development. Similar to other autoimmune diseases, the current dogma proposes that several genetic susceptibility genes in certain environments trigger the immune system to attack ‘self-antigens’, in this case the pigmentary system, resulting in the development of NSV. It should be noted that the exact mechanism of melanocyte loss by autoimmunity is still unclear, even though many studies have pointed out that cellular immunity plays an important role in the pathogenesis of NSV.

Previous studies have focused on determining the different types of immune cells in the local and peripheral compartment, which are involved in this process. Immunohistochemical studies indicated that both $CD4^+$ and $CD8^+$ T cells were detected in the perilesional skin of NSV, with decreased CD4/CD8 ratio (Ongenae et al., 2003). $CD8^+$ T cells isolated from peripheral blood and perilesional skins of NSV patients were reactive to melanocytes antigen-specific stimulation and were cytotoxic to melanocytes (Van Den Boorn et al., 2009; Wankowicz-Kalinska et al., 2003). Furthermore, analysis of the broad spectrum of cytokines produced by perilesion-derived $CD4^+$ and $CD8^+$ T cells confirmed polarization toward a type-1-like phenotype in both $CD4^+$ and $CD8^+$ compartments, which paralleled the depigmentation process observed clinically. T-cell infiltrates are commonly observed in progressive NSV; NSV patients have been reported to have increased numbers of peripheral T cells, which were reactive to melanocyte differentiation antigens, including tyrosinase, gp100, and melanoma antigen recognized by T cells (MART-1), compared to healthy donors (Mandelcorn-Monson et al., 2003; Palermo et al., 2001). Disappearing melanocytes were found to be co-localized with $CD8^+$ T cells, and they were demonstrated to express the skin homing and T-cell activation markers, perforin, and granzyme B (Le Poole et al., 1996; Van Den Wijngaard et al., 2000). A recent study further substantiated the role of T cells in NSV by showing melanocyte-specific cytotoxic activity of perilesional T cells in NSV patients (Van Den Boorn et al., 2009). However, melanocyte-reactive $CD8^+$ T cells are also found in healthy controls, suggesting that autoimmune reactivity is kept in check in healthy controls, and this modulation is defective in NSV (Visseren et al., 1995).

The mechanisms underlying the induction of autoreactive T cells and the loss of tolerance to melanocyte antigens have not been fully elucidated. Regulatory T (Treg) cells are known to inhibit autoreactivity and keep autoimmune responses in check. Different populations of Treg cells have been described, including thymically derived $CD4^+CD25^+FoxP3^+$ Treg cells and natural killer T (NKT) cells. Accumulating data indicate that a deficiency or dysfunction of $CD4^+CD25^+FoxP3^+$ Tregs may cause either systemic or organ-specific autoimmune diseases. Recently, Klarquist et al. (2010) reported that the number and function of

circulating Tregs in NSV were comparable to that of healthy controls, but NSV patients had reduced skin homing functional Treg. Interestingly, more recently, Ben Ahmed et al. (2012) reported the involvement of a functional defect of peripheral $CD4^+CD25^+$ Treg cells in NSV patients, thus the precise role of $CD4^+CD25^+FoxP3^+$ Treg cells in the pathogenesis of NSV warrants clarification.

Natural killer T (NKT) cells are another type of regulatory T cells that bridge the innate and adaptive immune systems. NKT cells are a heterogeneous subpopulation of T lymphocytes that co-express T-cell receptor (TCR) and NK lineage markers such as CD16, CD56, CD57, CD94, and CD161. Unlike conventional T cells, NKT cells recognize glycolipid antigens in the presence of the MHC class I-like antigen-presenting molecule CD1d (Linsen et al., 2005a). Type I classical or invariant NKT (*i*NKT) cells have a highly restricted TCR repertoire as they express an invariant Vα24-Jα18 rearranged TCRβ chain in humans, typically co-expressed with Vβ11-containing β chain; they can be identified by CD1d tetramers loaded with α-galactosylceramide (α-GalCer). The most remarkable property of *i*NKT cells is their ability to rapidly produced large amount of cytokines, such as IFN-γ and IL-4 (Linsen et al., 2005a; Matsuda et al., 2008). Consequently, *i*NKT cells have been shown to play crucial roles in a broad range of diseases, including infectious diseases, autoimmunity, and cancer (Bendelac et al., 2007; Wu and Van Kaer, 2009). A deficiency or dysfunction in *i*NKT cells has been found in different autoimmune diseases in human and mice, including rheumatoid arthritis, systemic lupus erythematosus, and type 1 diabetes (Bendelac et al., 2007; Cho et al., 2011; Laloux et al., 2001; Mars et al., 2004). It should be noted that many autoimmune diseases are epidemiologically associated with NSV, which imply the potential involvement of *i*NKT cells in the pathogenesis of NSV. However, there has been no *i*NKT cell studies reported in NSV so far.

In this study, we evaluated the general immune phenotypes, including the percentages of naïve and memory CD4 and CD8 T cells and their cytokine production, and the percentages of circulating $CD4^+CD25^+FoxP3^+$ Treg cells and *i*NKT cells as well as *i*NKT cytokine secretion functions, in a cohort of 43 NSV patients with active disease, and the results were compared to those obtained from race-, gender-, and age-matched healthy individual controls.

Results

Normal distribution of conventional T cells in the PBMCs of NSV patients

The immunophenotypes of circulating conventional T cells in our NSV patients were first characterized by flow cytometry. As shown in Figure 1A, B, the percentage of $CD4^+$ T cells (41.94 ± 9.14%) and $CD8^+$ T cells (25.26 ± 7.67%) in peripheral blood mononuclear cells (PBMCs) of NSV patients were comparable to healthy controls (41.90 ± 8.04% for $CD4^+$ and 28.70 ± 7.53% for $CD8^+$ cells, respectively), and the ratios of CD4/CD8 were also not significantly different (data not shown). The percentage of memory ($CD45RO^+$) and naïve ($CD45RA^+$) $CD4^+$ and $CD8^+$ cells were then evaluated (Figure 1C, D), and no significant difference was identified comparing patients with healthy controls ($CD4^+CD45RO^+$, 50.15 ± 16.82% versus 55.56 ± 11.32%; $CD4^+CD45RA^+$, 34.74 ± 15.35% versus 32.70 ± 10.45%; $CD8^+CD45RO^+$, 24.57 ± 10.10% versus 25.00 ± 10.24%; $CD8^+CD45RA^+$, 56.33 ± 17.10 versus 56.08 ± 16.20%, respectively, $P > 0.05$). T-cell activation based on CD69 expression was further analyzed. As shown in Figure 1E, F, no significant differences ($P > 0.05$) were observed in the percentages of $CD4^+$ $CD69^+$ (1.74 ± 1.46%) and $CD8^+CD69^+$ (5.32 ± 5.12%) T cells from NSV, compared to the percentages of $CD4^+CD69^+$ (1.85 ± 1.4%) and $CD8^+CD69^+$ (4.68 ± 4.04%) T cells from healthy controls. Thus, NSV patients from our large cohort had normal distribution and activation of conventional T cells in PBMCs.

No alteration in the frequencies of $CD4^+Foxp3^+$ Treg cells in the PBMCs of NSV patients

We next addressed whether the percentage of Treg cells in PBMCs was altered in NSV patients compared to healthy controls. PBMCs were stained with antihuman CD4, CD25, and Foxp3 antibodies. Lymphocytes gated on $CD4^+$ T cells were analyzed for the expression of combined CD25 and FoxP3 or FoxP3 expression alone (Figure 2A). Non-segmental vitiligo patients had no significant difference in the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells (3.69 ± 2.26%) compared with healthy controls (3.85 ± 1.62; $P > 0.05$). Furthermore, no difference was found in the percentage of $CD4^+Foxp3^+$ Treg cells between NSV patients and controls (5.68 ± 2.87% versus 5.87 ± 3.97%; $P > 0.05$) (Figure 2B). Additionally, when the ratio of memory/activated T cells ($CD4^+CD45RO^+$ cells) to $CD4^+Foxp3^+CD25^+$ Treg cells was analyzed in NSV patients and controls, no significant difference was observed (15.87 ± 5.69% in controls and 15.50 ± 7.66% in NSV patients, $P > 0.05$). Thus, the percentage of Treg cells in PBMCs was not altered in NSV patients.

Cytokine-producing T cells in the peripheral blood are not altered in NSV patients

Previous work has suggested a possible role for Th1 cells and inflammatory cytokine IFN-γ in the development of NSV in humans as well as in mice (Gregg et al., 2010; Van Den Wijngaard et al., 2000). We then analyzed the percentages of IL4- and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in PBMCs. As shown in Figure 3A, B, there were no significant differences in the percentages of IFN-γ^+CD4^+ (8.02 ± 4.65% in NSV and 9.35 ± 6.71% in control; $P > 0.05$) and IFN-γ^+CD8^+ T cells (28.88 ± 12.77% in NSV and 35.72 ± 15.44% in controls; $P > 0.05$), or $IL4^+CD4^+$ (2.23 ± 2.17% in NSV and 2.44 ± 1.93% in controls; $P > 0.05$) and $IL4^+CD8^+$ T cells (1.74 ± 1.26% in NSV and 1.62 ± 1.46% in controls; $P > 0.05$) between NSV and controls. Thus, NSV patients had comparable IL4- and IFN-γ-producing T cells in PBMCs compared to that from healthy controls.

Reduced frequency of circulating *i*NKT cells in NSV patients

Invariant natural killer T cells have been shown to play crucial roles in the development of human autoimmune diseases (Bendelac et al., 2007; Wu and Van Kaer, 2009). αGalCer-loaded CD1d tetramer is the best reagent currently available to accurately identify human *i*NKT cells in terms of specificity and sensitivity (Berzins et al., 2011; Lee et al., 2002b). The percentage of peripheral blood *i*NKT cells was determined for the 43 NSV patients and 43 healthy individuals using CD1d-loaded αGalCer tetramers. As shown in Figure 4, the percentage of *i*NKT cell in PBMCs from both NSV patient and healthy individual groups varied widely, ranging from 0.001 to 0.58%. However, the percentage of *i*NKT cells in NSV patients (0.065 ± 0.015%) was significantly reduced compared to that in healthy controls (0.108 ± 0.02%, $P = 0.0359$). This result suggests that defective *i*NKT cells may be involved in NSV development.

Phenotypes of *i*NKT in NSV patients

Mature human *i*NKT cells can be divided into functionally distinct $CD4^+$ and $CD4^-$ two subsets and CD69 was used as a marker characterizing *i*NKT cell maturation and activation status. To investigate whether *i*NKT cells from NSV patients exhibited phenotypic abnormalities, we first analyzed the percentages of $CD4^+$ *i*NKT subset and $CD69^+$ *i*NKT cells in PBMCs. Figure 5A shows the representative dot plots from the analysis of $CD4^+$ subset and $CD69^+$ *i*NKT cells. As shown in Figure 5B, there were no significant differences detected between NSV patients and healthy controls. However, *i*NKT cells from NSV patients exhibit a trend toward decreased $CD4^+$ subset ($P = 0.06$). Activated *i*NKT cells release large amounts of cytokines, including IL-4 and IFN-γ. As shown in Figure 5C, there was a strong induction of IFN-γ and IL-4 from activated *i*NKT cells. However, the percentages of IFN-γ- and IL-4- producing *i*NKT cells from both NSV patients and control

groups varied widely, and no significant difference was identified in the capacity of peripheral *i*NKT cells cytokine secretion between NSV patients and healthy controls (Figure 5D).

Discussion

Autoimmune diseases are characterized by failure in the mechanisms of tolerance to self-antigens. Immunoregulation by Treg cells, including $CD4^+FoxP3^+CD25^+$Tregs and *i*NKT cells, has emerged as an important part of peripheral tolerance. Studies from our laboratory and others suggest that defects in the number and function of Treg cells contribute to autoimmune disease development (Ly et al., 2006; Zhou et al., 2011). Although the pathogenesis of NSV is still not yet fully understood, accumulated studies suggest that NSV is an autoimmune disease (Le Poole and Luiten, 2008; Spritz, 2011). The present study represents, to our knowledge, the first large-scale systemic analysis of basic immunophenotypes, from conventional T cells to Treg cells, in NSV patients with strictly matched healthy individual controls. We found that there were no differences in the percentages of conventional CD4/CD8, naïve or memory/active CD4/CD8 T cells, or their Th1/Th2 cytokine production, and that the percentages of $CD4^+CD25^+$ $FoxP3^+$Treg cells were comparable between NSV patients and healthy controls. However, the percentages of peripheral *i*NKT cells were significantly decreased in NSV patients, suggesting the possible involvement of *i*NKT cells in the autoimmunity of NSV.

Previous studies indicated the potential abnormal subsets of circulating T cells in NSV (Basak et al., 2008; Mahmoud et al., 2002; Pichler et al., 2009). Mahmoud et al. (1998, 2002) reported that NSV patients from Japanese population had elevated percentages of memory ($CD4^+CD45RO^+$) T cells and decreased percentages of naive T cells ($CD4^+CD45RA^+$) compared to healthy controls, supporting the hypothesis of T-cell activation as a major feature of the disorder. Interestingly, we did not find the significant differences in our NSV patients compared to healthy controls. This may be related to different genetic background of patient and control subjects. The percentages of $CD4^+CD25^+$Treg cells in peripheral blood in most autoimmune diseases investigated to date have either been normal or decreased. On analyzing peripheral $CD4^+CD25^+FoxP3^+$Treg cells in 13 NSV patients, Klarquist found no differences in the percentage and immune suppression function between NSV patients and controls, but $CD4^+CD25^+FoxP3^+$Treg cells from NSV patients had defects on skin homing (Klarquist et al., 2010). More recently, Ben Ahmed et al. also reported the comparable frequency of Tregs ($CD4^+CD25^{high}$) between NSV (10 patients) and controls, but they stated that the suppressive function of $CD4^+CD25^{high}$ Treg cells was decreased in four progressive NSV patients (Ben Ahmed et al., 2012). Nonetheless, it is now recognized that $CD25^+$ alone is not the best marker for detecting $CD4^+$Tregs, especially for individuals with progressive inflammatory diseases, in which $CD4^+CD25^{high}$Tregs could potentially be contaminated with activated $CD4^+$ T cells. In our study, we analyzed $CD4^+CD25^+FoxP3^+$Treg cells in 43 progressive NSV. Our results further support previous findings (Ben Ahmed et al., 2012; Klarquist et al., 2010) that the percentages of peripheral $CD4^+CD25^+FoxP3^+$Treg cells were not altered in NSV patients. However, the suppressive function of $CD4^+CD25^+FoxP3^+$Tregs remains to be validated in future studies.

Another key immunoregulatory T cell is the *i*NKT cell. Understanding of the role of *i*NKT cells in the development of NSV is lacking. We used CD1d-tetramer staining, a specific method for *i*NKT identification, to accurately determine the percentage and function of *i*NKT cells. A significant decrease in peripheral blood *i*NKT cells was observed in NSV patients. Mahmoud previously reported that peripheral NKT cells ($CD3^+$ $CD16^+CD56^+$) were significantly reduced in NSV patients compared to healthy controls (Mahmoud et al.,

2002). Even though the NKT cells in their analysis contained both *i*NKT cells and other non-*i*NKT cells, our results further support their findings. Although *i*NKT cells constitute only a small fraction of lymphocytes, their ability to rapidly secrete large amounts of cytokines, including IFN-*γ*, IL-4, IL-10, and IL-13, makes them an important regulator of the Th1/Th2 cytokine balance in immune responses. Human *i*NKT cells segregate into $CD4^+$ and $CD4^-$ subsets with distinct phenotypic and functional characteristics (Gumperz et al., 2002; Kim et al., 2002; Lee et al., 2002a). $CD4^+$ *i*NKT cells produce both Th1 and Th2 cytokines, whereas the $CD4^-$ subset exhibits a Th1 cytokine profile. We showed here that *i*NKT cells in NSV exhibited a trend of decreased $CD4^+$ subset, even though the difference was not statistically significant. Significant progress has been made regarding the role of *i*NKT cells in the pathogenesis of human autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, type 1 diabetes (T1D), systemic lupus erythematosus, and adrenoleukodystrophy (Gautron et al., 2010; Illes et al., 2000; Kojo et al., 2001; Linsen et al., 2005b; Mars et al., 2004; Sumida et al., 1995; Van Der Vliet et al., 2001), suggesting a protective role of *i*NKT cells. We and others have previously shown that deficiencies in *i*NKT cell number and function mediate the development of T1D in nonobese diabetic (NOD) mice, and that *α*GalCer-induced NKT cell activation corrected these deficiencies and reduced the incidence of spontaneous and recurrent diabetes in NOD mice (Mi et al., 2003, 2004; Sharif et al., 2001). In studies using T1D mouse model, it has been demonstrated that Th1 cell-mediated tissue damage was initially regulated by *i*NKT cells and the protection conferred by *i*NKT cells was associated with a Th2 shift within the pancreatic islets, and IL-4 has also been implicated as a key mediator of immunoregulation (Hammond et al., 1998; Laloux et al., 2001). It has been shown that psoriasis is associated with decreased numbers of circulating *i*NKT cells (Koreck et al., 2002; Van Der Vliet et al., 2001), and a decreased percentage of $CD3^+CD56^+$ population of *i*NKT cells in patients with psoriasis tended to be even lower in those patients with frequently relapsing and treatment-resistant disease (Koreck et al., 2002). In multiple sclerosis, the critical role of *i*NKT phenotype in disease pathogenesis was demonstrated by the strong Th2 bias of the $CD4^+$ *i*NKT cell lines from patients in remission compared with that from patients in relapse (Araki et al., 2003). Thus, taken together, the decreased peripheral blood *i*NKT cell percentage and the trend toward changed *i*NKT cell phenotype suggest a protective and immune regulatory role of *i*NKT cells in the pathogenesis of NSV.

As is the case for the association of *i*NKT cells with multiple other autoimmune diseases, key unanswered questions regarding the role of *i*NKT cells in NSV include whether *i*NKT cell deficiencies predispose individuals to disease and what the cause(s) of *i*NKT cell deficiencies is (are). Our current analysis has certain limitations; for example, in *i*NKT cell function analyses, phorbol myristate acetate (PMA) and ionomycin activated *i*NKT cells through bypass of the TCR upstream signal, which cannot elucidate whether *i*NKT cells responding to their TCR through a-GalCer are defective, including *i*NKT cell proliferation and cytokine production. To gain a better understanding of the significance of *i*NKT cell defects in the pathogenesis of NSV, a more comprehensive characterization of *i*NKT cells from blood, especially during both the relapse and remission phases of disease, will be needed. Studying the role of *i*NKT cells in skin lesions may further uncover how *i*NKT cells contribute to disease pathogenesis locally. However, only very few infiltrating cells could be isolated from vitiligo skin lesions; with the known low frequency of *i*NKT cells, it was not possible to accurately perform FACS analyses for *i*NKT cell number and subset, and to assess their cytokine production.

Using an *i*NKT cell-deficient mouse model, a recent study demonstrated that *i*NKT cells play an important role in limiting the development of the Th17 lineage, suggesting that *i*NKT cells provide a natural barrier against Th17 responses (Mars et al., 2009). Interestingly, additional studies indicate that in patients with NSV, serum IL-17 levels were

positively correlated with the extent of body area involvement (Basak et al., 2009) and that Th17 cells were increased in NSV lesions (Bassiouny and Shaker, 2011; Kotobuki et al., 2012; Wang et al., 2011). Thus, *i*NKT cell deficiency in NSV may not only contribute to Th1 and autoreactive CD8 T cells, but also to dysregulated Th17 cells. Taken together, our data suggest that *i*NKT cells may play a role in the pathogenesis of NSV. As shown in Figure 6, both *i*NKT cells and $CD4^+Foxp3^+$ Treg cells may work together to regulate autoreactive Th1, Th17, and $CD8^+$ T cells during NSV development. Additional studies are clearly needed to further test this hypothesis.

Methods

Patients

The study cohort included 43 progressive NSV patients and 43 age-, gender-, and race-matched healthy controls. Patients with NSV were recruited with the following criteria: (i) a clinical diagnosis of generalized NSV with related criteria (Taieb and Picardo, 2007), (ii) discontinuation of any systemic therapy for at least 4 weeks, and (iii) development of new lesions within the past 3 months. Clinical characteristics of the patients are summarized in Table 1. The study was approved by the Institutional Review Board of Henry Ford Health System, and written informed consent was obtained from all participants.

T-cell stimulation

Peripheral blood mononuclear cells (2×10^6/ml) were cultured in RPMI 1640 medium in 24-well plate and stimulated for 5 h at 37°C with 50 ng/ml of PMA and 1 μmol/ml of Ionomycin in the presence of 0.67 μl/ml Golgi stop (BD Bioscience, San Jose, CA, USA). After incubation, cells were collected for flow cytometry analysis.

Flow cytometry analyses

To analyze CD4, CD8, Tregs, and *i*NKT cell frequencies and phenotypes, PBMCs were stained with the following antihuman antibodies: anti-CD3 (clone SK7), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anit-CD25 (M-A251), anti-CD69 (FN50), CD45RA (HI100), CD45RO (UCHL1), and CD1d-αGalCer tetramers (NIH Tetramer Facility). For intracellular Foxp3 and cytokine staining, PBMCs were fixed and permeabilized and with fixation/permeabilization solution, and then stained with antihuman IFN-γ (4S.B3), IL-4 (MP4-25D2), and Foxp3 (M-A251). All antibodies were purchased from BD Biosciences or eBioscience. Stained cells were analyzed either on a FACSCalibur or FACSAriaII (BD Bioscience) using FlowJo software (Tree Star Inc, Ashland, OR, USA). To make the data from patients and healthy controls maximally comparable, patient and matched control PBMCs were activated and then stained with antibodies and analyzed side by side.

Statistical analysis

Data are presented as mean ± SD, frequencies, and percentages as appropriate. For data that were not normally distributed, comparison of quantitative variables between the study groups was performed using the Mann–Whitney *U* test. For all cases, $P < 0.05$ was considered significant. All statistical calculations were performed using the Prism program (GraphPad, La Jolla, CA, USA).

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Significance

The present study represents, to our knowledge, the first large-scale systemic analyses of peripheral T-cell immunophenotypes, from conventional T cells to regulatory T cells (Tregs), in a large cohort of progressive non-segmental vitiligo (NSV) patients. Our results show that there are no significant differences in the basic immunophenotypes of conventional CD4/CD8 T cells and the percentages of $CD4^+CD25^+FoxP3^+$ Tregs between NSV patients and healthy controls. However, the percentages of peripheral invariant natural killer T (iNKT) cells, which have been thought to play a major role in autoimmune diseases, were significantly decreased in NSV patients. Thus, our results highly suggest that iNKT cells may be involved in the immnuopathogeneis of NSV.

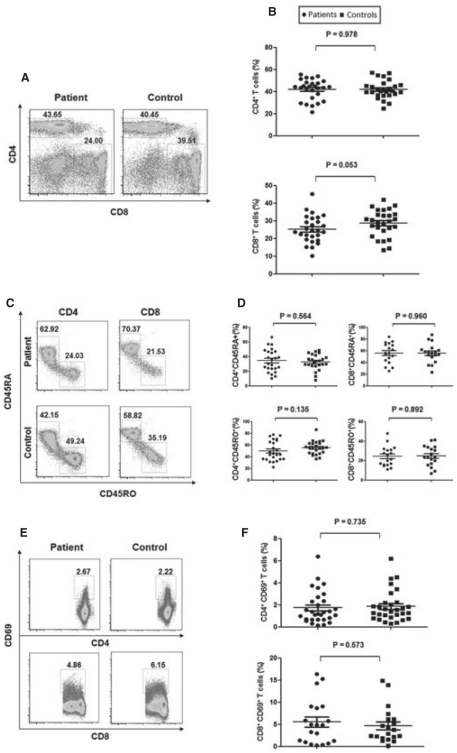

Figure 1.

Distribution of conventional T cells in the peripheral blood. (A) Flow cytometry analysis of $CD4^+$ and $CD8^+$ T cells in peripheral blood mononuclear cells (PBMC) from non-segmental vitiligo (NSV) patients and healthy controls. (B) Summary plots showing individual results of the frequency of $CD4^+$ and $CD8^+$ T cells described. (C) Flow cytometry analysis of memory ($CD45RO^+$) and naïve ($CD45RA^+$) $CD4^+$ and $CD8^+$ T cells in PBMCs. (D) Summary plots showing individual results of the frequency of $CD45RA^+$ and $CD45RO^+$ $CD4^+$ and $CD8^+$ T cells in NSV patients versus healthy controls. (E) Representative FACS dot plots for CD69 expression in $CD4^+$ and $CD8^+$ T cells. (F) Summary plots showing individual results of the frequency of $CD69^+$ $CD4^+$ T cells and $CD69^+$ $CD8^+$ T cells in NSV patients versus healthy controls.

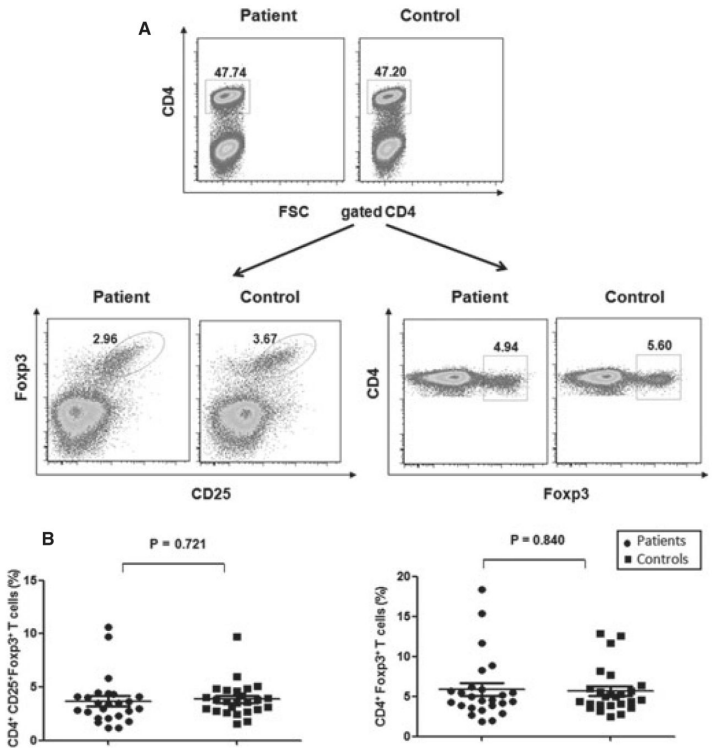

Figure 2.

Flow cytometry analysis of peripheral blood $Foxp3^{+}CD4^{+}$ Treg cells. (A) Peripheral blood mononuclear cells (PBMCs) were stained with anti-CD4, CD25, and Foxp3. Gated $CD4^{+}$ lymphocytes were selected for $CD25^{+}Foxp3^{+}$ or $Foxp3^{+}$ cell analyses, respectively. (B) Summary plots showing individual results of the frequency of $CD25^{+}Foxp3^{+}CD4^{+}$ and $Foxp3^{+}CD4^{+}$ in non-segmental vitiligo (NSV) patients versus healthy controls.

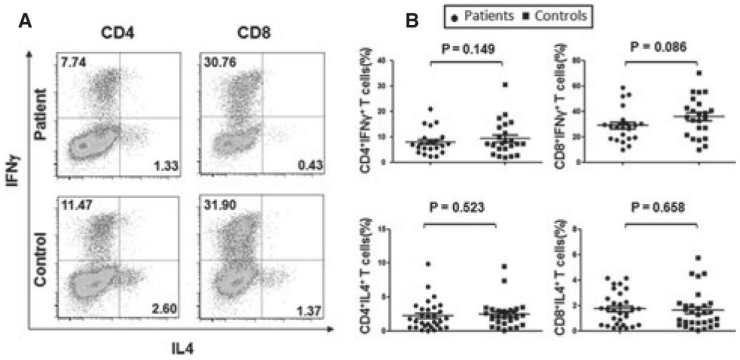

Figure 3.

Cytokine-producing T cells from peripheral blood. Peripheral blood mononuclear cells (PBMCs) isolated from non-segmental vitiligo (NSV) patients and healthy controls were stimulated with phorbol myristate acetate (PMA) and ionomycin for 5 h in the presence of Golgi stop. Then, the IFN-γ- and IL-4-producing T cells were determined by intracellular staining and flow cytometry analysis. (A) Representative FACS dot plots for IL-4- and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells gated on CD4-positive and CD8-positive cells. (B) Summary plots showing individual results of the frequency of IL4- and IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in NSV patients versus healthy controls.

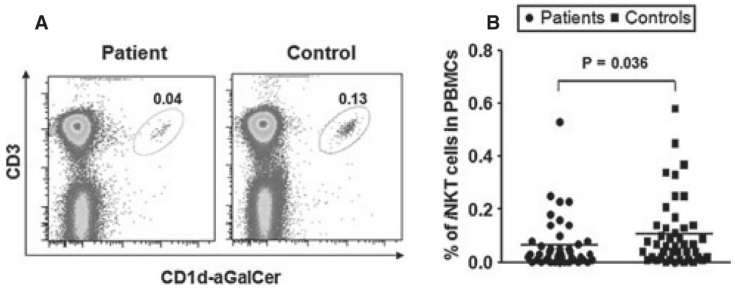

Figure 4.

Invariant natural killer T (*i*NKT) cell frequency in peripheral blood mononuclear cells (PBMCs) of non-segmental vitiligo (NSV) patients. Peripheral blood mononuclear cells were separated by Ficoll gradient, and flow cytometry was performed using CD1d-aGalCer tetramers and anti-mouse CD3 antibody. (A) Representative FACS dot plots for *i*NKT cells from NSV patient sand healthy controls. (B) Summary plots showing individual results of *i*NKT cell frequency in NSV patients versus healthy controls.

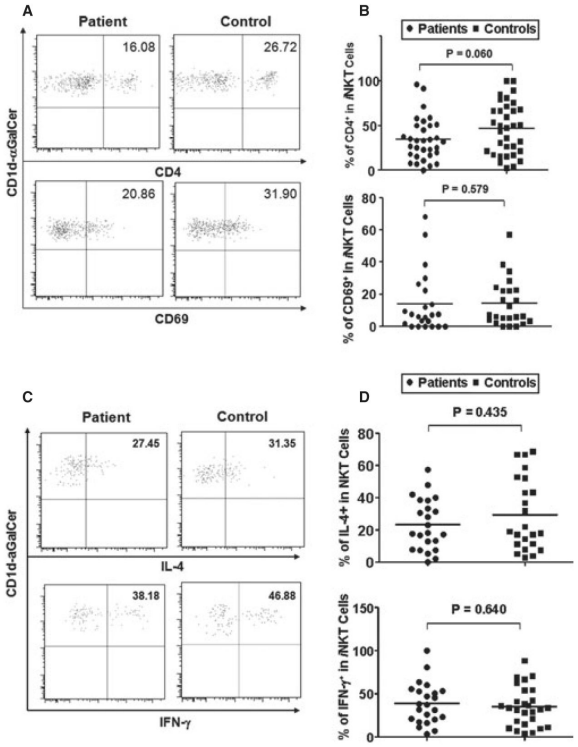

Figure 5.

Invariant natural killer T (*i*NKT) cell subsets, maturation, activation status, and cytokine production in non-segmental vitiligo (NSV) patients. Circulating *i*NKT cells were identified as CD1d-aGalCer tetramer^{+}CD3^{+} cells, and then their subsets, maturation, and activation were further determined by the expression of CD4 and CD69, respectively. Intracellular IFN-*γ* and IL-4 production of circulating *i*NKT cells was analyzed after stimulation with phorbol myristate acetate (PMA) plus ionomycin in the presence of GolgiStop for 5 h. (A) Representative FACS dot plots for *i*NKT cell CD4 and CD69 expression in NSV patients and healthy controls. (B) Summary plots showing individual results of the frequency of CD4^{+} *i*NKT cell and CD69^{+} *i*NKT cells in NSV patients versus healthy controls. (C) Representative FACS dot plots for IL-4 and IFN-*γ* expression in gated *i*NKT cells. (D) Summary plots showing individual results of the frequency of IL4- and IFN-*γ*-producing *i*NKT cells in NSV patients versus healthy controls.

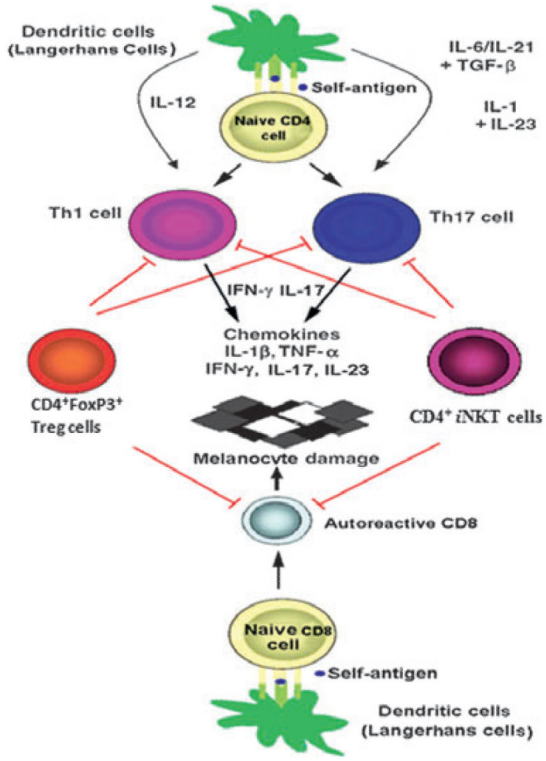

Figure 6.

A model for immunopathogenesis of autoimmune nonsegmental vitiligo (NSV). Dendritic cells (Langerhans cells in epidermis) present self-antigen to activate naïve $CD4^+$ and $CD8^+$ T cells and induce naïve $CD4^+$ T cells to differentiate into Th1 and Th17, which mediate the destruction of melanocytes. $CD4^+CD25^+Foxp3^+$ Tregs and invariant natural killer T (*i*NKT) cells can block these processes through the secretion of protective cytokine (IL-4 and IL10) or cell–cell contact to suppress Th1, Th17, and autoreactive $CD8^+$ T-cell function. Defective frequencies and functions of $CD4^+CD25^+Foxp3^+$ Tregs and *i*NKT cells, with up-regulated autoreactive Th1, Th17, and $CD8^+$ T cells, contribute to immune destruction of melanocytes in autoimmune NSV.

Table 1

Clinical characteristics of NSV patients and healthy controls

Characteristics	Healthy controls (n = 43)	NSV patients (n = 43)
Sex (male/female)	15/28	15/28
Age, median (range)	40 (18–71)	45 (18–72)
Autoimmune diseases (positive/negative)	ND	13/30
Family history of NSV (positive/negative)	ND	16/27
Percent of body surface area involved, Median (range)	ND	15 (5–100)

NSV, non-segmental vitiligo.