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Accepted Date : 20-Dec-2013

Article type : Original Article

## **IFN- $\gamma$ /CCR5 expression in invariant NKT cells and CCL5 expression in capillary veins of dermal papillae correlate with development of psoriasis vulgaris**

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Short title: IFN- $\gamma$ , CCR5, and CCL5 expression in psoriatic skin

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*Grant Support:* None.

*Disclosures:* All authors declare that there are no conflicts of interest with respect to this paper or the material it contains.

### **What's already known about this topic?**

- NKT cells are present in psoriasis plaques.
- Injection of NKT cells into transplanted psoriatic skin drives lesion formation.
- Imbalance of IFN- $\gamma$  and IL-4 contributes to lesion development.
- Injection of IL-4 into the plaque ameliorates psoriasis.

### **What does this study add?**

- The phenotype of invariant NKT (iNKT) cells in psoriasis is clarified.

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- iNKT cell number correlates with length of the rete ridge.
- iNKT cell number correlates with the psoriasis area and severity index, epidermal hyperplasia and degree of microabscess formation.
- iNKT cells in psoriatic skin have higher CCR5 expression.
- The CCR5+ iNKT cell count correlates with that of CCL5+ capillaries in dermal papillae.
- CCL5+ capillary endothelial cell count correlates with epidermal hyperplasia.

## **Abstract**

### **Background**

There have been extensive studies regarding which types of T lymphocytes are involved in psoriasis vulgaris (PV). However, it has remained unclear as to which types of T lymphocytes may directly contribute to psoriasiform epidermal and vascular hyperplasia.

### **Objectives**

To understand the role of TCRV $\alpha$ 24+ invariant natural killer T (iNKT) in the development of PV, a total of 17 patients with were enrolled in this study.

### **Methods**

In the present study, using biopsy samples of PV plaques, TCRV $\alpha$ 24<sup>+</sup> invariant natural killer T (iNKT) cells were investigated regarding cytokine production to understand their roles in development of disease.

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## **Results**

The number of IFN- $\gamma$ + iNKT cells correlated with the length of the psoriasiform hyperplasia rete ridge and the psoriasis area and severity index (PASI). IFN- $\gamma$ + iNKT cells in psoriatic skin exhibited higher C-C chemokine receptor type 5 (CCR5) expression, and the amount of C-C chemokine ligand 5 (CCL5), a ligand for CCR5, was increased in capillary veins of psoriasis plaques. CCR5+ iNKT cell numbers significantly correlated with the number of capillary vein endothelial cells expressing CCL5 in PV. Furthermore, the number of CCL5+ capillary veins correlated with the maximal rete ridge length.

## **Conclusions**

IFN- $\gamma$ /CCR5 expression in iNKT cells and CCL5 expression in dermal papillae vessels of dermal papillae are correlated with the development of psoriasiform hyperplasia and microabscess. We propose that these iNKT cells may become useful targets for development of novel therapeutic approaches to PV.

## **Text**

Whether psoriasis vulgaris (PV) is an epithelial or immunological disorder has been debated. Recently, the pathogenesis has been investigated by looking at cytokine production in epidermal and dermal papillae lesions. PV is caused by an immune response driven by high levels of interferon (IFN)- $\gamma$  secreted by helper T type 1 (Th1) cells<sup>1</sup>. Thus, the pathogenesis is definitely associated with a cytokine-producing environment. However, whether other types of T cells, aside from conventional Th1 cells, actually secrete and contribute to the pathogenesis remains unclear.

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Whereas the role of several specific T cell types, such as T helper (Th) 17, has recently been investigated,<sup>1-3</sup> the role of natural killer T (NKT) cells has not been sufficiently identified, in spite of the fact that they are known to be resident in the plaques of PV<sup>4,5</sup>

Among NKT cells, invariant type NKT cells (iNKT) have an invariant T cell receptor that is restricted to CD1d molecule.<sup>6-8</sup> In addition to T helper (Th) 1 cytokines, such as IFN- $\gamma$ , iNKT cells can produce anti-inflammatory Th2 cytokines, such as interleukin (IL)-4.<sup>6-9</sup> iNKT cells have been implicated in the control of infections and inflammatory bowel disease,<sup>10</sup> transplantation tolerance or rejection,<sup>11,12</sup> spontaneous abortion,<sup>13</sup> suppression of diabetes,<sup>14</sup> and regulation of allergic disorders.<sup>15-17</sup> In fact, iNKT cells have been considered to play pivotal roles in the development and/or healing of PV, and experimental studies have shown that injection of human cells having NKT characteristics into transplanted psoriatic skin could drive lesion development.<sup>18</sup> In the previously diseases listed, iNKT cells are activated in a CD1d dependent fashion through lipid antigen-presentation.<sup>6-8</sup> iNKT cells are activated in a CD1d-dependent fashion through lipid antigen presentation.<sup>6-8</sup> A recent study demonstrated that this activation is associated with the inflammatory changes involving the Toll-like receptor pathway in the innate immune system.<sup>19</sup> For the present study, the necessary histological techniques for multicolour histopathologic analyses were developed for this application.<sup>12,20</sup>

We focused on the capillary hyperplasia in the dermal papillae that has been one of the critical pathologic findings of PV, as referenced in the Psoriasis Area and Severity Index (PASI). During the development of a skin injury, the interaction between CCL5 and its receptor, CCR5, has become one of the novel molecular targets for modulation of neovascularization in various skin wounds.<sup>21,22</sup> However, their roles in PV have not been identified, and the effector cells that produce the CCL5 have not been sufficiently identified. In the present study, we aimed to clarify the role of the

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interaction between CCL5 and CCR5 in PV and to determine which types of cells produce CCL5. A previous study showed that CD56<sup>bright</sup> CD16<sup>(-)</sup> natural killer cells accumulate in psoriatic skin in response to CXCL10 and CCL5 and exacerbate skin inflammation.<sup>23</sup> Accordingly, we investigated the question of whether iNKT cells produce CCL5.

## **Patients and methods**

### **Subjects and study design**

A total of 17 patients with PV and control 17 patients with atopic dermatitis (AD), were enrolled in this study. Normal skin data was obtained from autopsy samples of a patient whose family was informed of this research use. Clinical details of enrolled patients are summarized in Tables 1 & 2. Informed consent and permission to harvest tissues for the study were obtained from individual patients according to guidelines of the Ethics Committee of Kyoto University.

### **Histological evaluation of NKT cells**

Skin biopsies were collected from an indicator plaque from each patient. The biopsy samples were embedded in paraffin and 3- $\mu$ m-thick sections were stained with monoclonal antibodies specific for a total of 11 antigens, as follows: CD3, CD4, CD20, CD31, and CD56 (Dako, Gostrup, Denmark); IFN- $\gamma$ , IL-4, CCR5, TCRV $\alpha$ 7.2, and CD161 and TCRV $\alpha$ 24 (Immunotech, Marseille, France). Antibody binding was visualized by a catalyzed signal amplification (CSA) kit (Dako, Glostrup, Denmark) with 3, 3'-diaminobenzidine (DAB) chromogen, or with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) (Vector Laboratories, Burlingame, CA, USA) as

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fluorescence chromogens. The detailed protocol was reported previously by us.<sup>24</sup> iNKT cells positive for IFN- $\gamma$ , IL-4, or CCR5 and capillary vessels positive for CCL5 were counted in 5 fields (400x) from each of the 17 individual PV patients, the 17 AD control subjects, and in normal skin. The number of iNKT cells in biopsy samples was determined by three investigators who were blinded to the source of clinical data. Counts of individual cells were measured automatically using Meta Series Software 7.5.4 (Molecular Devices, Downingtown, PA, USA).<sup>19</sup> For each slide, the mean number of iNKT cells was calculated using 600x magnification.<sup>14</sup>

### **Statistical analysis**

Comparisons were made using the Student's *t*-test after confirmation with the F-test that the parameter was normally and equally distributed. Calculations, including regression analysis, for comparisons of histopathologic and clinical parameters, such as the PASI, were performed using StatView-J 5.0 software (SAS Institute, Cary, NC, USA). A value for  $P \leq 0.05$  was considered significant.

## **Results**

### **iNKT cell infiltrates in psoriasis**

Assessed characteristics of tissue samples included extent of epidermal thickness, epidermal apoptosis, parakeratotic scale formation, and degree of lymphocyte and leukocyte infiltration, for pathologic diagnosis of psoriasis (**Table 1 and Table 2**). The epidermal rete ridge and the thinnest epidermis were measured in the most prominent parakeratotic plaque, as shown in **Fig. 1a**. Within

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the dermal papillae, the capillary vein was dilated and surrounded by infiltrates of lymphoid cells. Immunohistochemical analysis successfully detected the TCRV $\alpha$ 24+ iNKT cell type in the psoriasis plaque, but not in normal skin (**Fig. 1b**). Combined staining showed that this cell type often expressed CD56 and CD161, NK cell markers. In addition, the T cell phenotypic markers CD3 and CD4 were positive in these NK marker-positive cells, indicating that these cells shared the natural killer T cell phenotype. TCRV $\alpha$ 24+ iNKT cells were counted automatically using Meta series software (see Methods section).<sup>12</sup> The iNKT cells had a lobated nucleus and more cytoplasm than other lymphocytes (**Fig. 1c**). The number of iNKT cells found in the dermal papillae was significantly higher than that seen in normal skin ( $P < 0.05$ ) (**Fig. 1d**).

As a result, among the histologic findings mentioned above, maximal rete ridge length (without the parakeratotic epidermal layer) was quantitatively correlated with iNKT cell count in PV ( $R^2 = 0.809$ ), but not in normal skin ( $R^2 = 0.005$ ) (**Fig. 2a**). In addition, the iNKT cell count correlated with the PASI ( $R^2 = 0.746$ ) (**Fig. 2b**), which correlated with the maximal rete ridge length ( $R^2 = 0.794$ ) (**Fig. 2c**).

### IFN- $\gamma$ + iNKT cells in psoriasis lesions

Recently, it has been demonstrated that NKT cells exist in the majority of AD lesions.<sup>16, 17</sup>

Therefore, we selected AD as an atypical sample to be a useful control.<sup>17</sup> To identify the cytokine-producing activity of infiltrating iNKT cells, staining of PV (**Fig. 3a**) or AD (**Fig. 3b**) specimens was performed with monoclonal antibodies specific for TCRV $\alpha$ 24 and IFN- $\gamma$ .

IL-4+□□iNKT cells were dominant in AD relative to PV ( $*P < 0.05$ ), and IFN- $\gamma$ +□□iNKT cells were dominant in PV relative to AD ( $**P < 0.05$ ) (**Fig. 3c**). Thus, the dominant iNKT cell phenotype

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was significantly different in PV relative to AD lesions. Furthermore, the number of IFN- $\gamma$ + iNKT cells correlated with the maximal length of the rete ridge ( $R^2 = 0.69$ ) and microabscess area length ( $R^2 = 0.76$ ) in PV specimens (**Fig. 3d, e**), suggesting that IFN- $\gamma$ + iNKT cells contribute to the histologic changes specific to PV lesions. In contrast, in PV lesions, the number of IL-4+ iNKT cells correlated with neither the maximal rete ridge length nor microabscess area length (**Fig. 3d, e**).

### **CCR5 expression in iNKT cells in psoriasis plaques**

CCR5 signals can facilitate activation-induced apoptosis of NKT cells as well as skew NKT cell production from IL-4 towards IFN- $\gamma$ .<sup>23</sup> In this study, immunohistochemical analyses demonstrated that iNKT cells were positive for CCR5 (**Fig. 4a**) and that capillary veins positive for CD31, a pan-capillary vessel marker, in the PV plaques were positive for CCL5 (**Fig. 4b**). iNKT cells positive for CCR5 were significantly increased in number in PV plaques (**Fig. 4c**). In addition, capillary endothelial cells positive for CCL5 were significantly increased in PV plaques (**Fig. 4d**). Furthermore, the number of CCL5-positive capillary veins correlated with the maximal length of the rete ridge ( $R^2 = 0.71$ ) (**Fig. 4e**), but not with the microabscess area length ( $R^2 = 0.07$ ) in PV specimens (**Fig. 4f**), suggesting that CCL5-positive capillary veins are related to the histologic changes of PV.

### **Discussion**

The current results regarding cytokine production in iNKT cells are compatible with those of previous studies of IL-4 and IFN- $\gamma$  secretion from T cells in PV.<sup>25</sup> Notably, IFN- $\gamma$ + iNKT cells

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may be involved in the inflammatory changes of psoriasis with microabscess. Probably, the released IFN- $\gamma$  is a trigger of inflammation that promotes epidermal hyperplasia and recruits neutrophils to the epidermis. As shown in Figure 3C, IL-4+ iNKT cells were also observed in the psoriasis plaque. Because the IL-4+ cell count did not correlate with histopathologic parameters, it remains unclear whether IL-4+ iNKT cells are related to the development of PV; alternatively, iNKT cells have the potential to suppress psoriasis by IL-4 release, according to a previous study.<sup>26</sup> iNKT cells thus may contribute to both the development and suppression of PV, as reported for lesional skin of atopic eczema.<sup>17</sup> Alternatively, the imbalance of cytokines produced by iNKT cells may be one of the triggers of PV.

Furthermore, the expression of CCR5 in iNKT cells was identified; these data are compatible with a previous report that CCR5 was expressed in plaque-infiltrating T cells.<sup>27</sup> In this study, a larger fraction of iNKT cells in plaques expressed CCR5 than did those present in normal skin. A pivotal role for CCL5/CCR5 interaction in the recruitment of endothelial progenitor cells during mouse wound healing followed by neovascularization consisting of capillary hyperplasia and granulomatous inflammation was previously demonstrated.<sup>21</sup> The current data suggest the possibility that iNKT cells contribute to psoriatic skin deterioration through CCL5/CCR5 interaction. In addition, inflammation involving CCL5 expression in the capillary veins may be caused by vascular abnormalities, or leaky remodelling in association with the rete ridge hyperplasia; however, the expression did not significantly correlate with microabscess degree (Figure 5E). This suggests that localized epidermal inflammation, such as microabscess, is not a sufficient trigger of capillary change. On the basis of these data and ideas, the contribution of iNKT cells to PV is summarized in Figure 5. Although it remains unclear why the expression profiles in

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capillary veins in psoriasis are similar to that in skin wounding,<sup>21, 22</sup> and more clinical studies are required, blocking CCR5 by the inhibitor is one of the therapeutic interventions for diminishing the inflammatory process.<sup>27</sup>

The applied multicolour fluorescent immunohistochemistry of iNKT cells was effective for the current study, and it may become a widely used tool for histological evaluation. We successfully enhanced the fluorescent signal with a combination of software for accurate morphologic histologic assessment (see Materials section).<sup>24</sup> Recent experimental studies have demonstrated that other cytokines, such as IL-17, IL-22, and IL-23, contribute to epidermal acanthosis.<sup>28-30</sup> In a future study, we will histologically examine the release of those cytokines by iNKT cells or Th17 cells, to ascertain whether these cells directly contribute to psoriasis hyperplasia.

In conclusion, IFN- $\gamma$ /CCR5 expressions in iNKT cells and CCL5 expression in capillary vessels of dermal papillae is correlated with the development of psoriasiform epidermal and dermal capillary hyperplasia. We propose that these iNKT cells may become useful targets for development of novel therapeutic approaches to PV, after the mechanisms leading to their activation have been fully elucidated.<sup>8, 21, 22</sup>

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## Figure Legends

### Figure 1. Psoriasis plaque with iNKT cell infiltration

(a) Measurement of length of rete ridge and epidermal thickness. Black vertical line: length of rete ridge containing psoriasiform hyperplasia (300  $\mu$ m) Blue line: epidermal thickness (80  $\mu$ m).

(b) Phenotypic analysis using double colour fluorescence staining of iNKT cells positive for TCRV $\alpha$ 24. TCRV $\alpha$ 24 was stained using PE-labelled anti-TCRV $\alpha$ 24 antibody (in red). Other phenotypic markers were analyzed by staining with FITC-labelled anti-CD56, anti-CD161, anti-CD3, or anti-CD4 antibodies (green). The bottom micrograph shows a merged image. TCRV $\alpha$ 7.2 staining was performed as a negative control for TCRV $\alpha$ 24 double staining. The

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micrograph at the bottom right shows no signal after PE-fluorescence anti-TCRV $\alpha$ 24 antibody staining of iNKT cells in normal skin (merged photos are shown) (600 $\times$  magnification). FITC, fluorescein isothiocyanate.

(c) The micrograph shows one DAB-stained iNKT cell labelled with anti-TCRV $\alpha$ 24 antibody. The right photo shows staining of FITC-labelled CD20 (a B cell marker) and PE-labelled TCRV $\alpha$ 24.

(d) iNKT cell counts in psoriasis plaque, AD and normal skin. Mean  $\pm$  SD. \* $P=0.02$ , psoriasis vs. normal skin. HPF: high power field (600 $\times$  magnification).

### **Figure 2. iNKT cell counts and length of rete ridge**

(a) A regression analysis showing relationship between maximal length of rete ridge and iNKT cell count, in PV (left), and normal skin (right).

(b) A regression analysis of PV samples, showing significant correlation between iNKT cell count and PASI.

(c) A regression analysis of PV samples, showing significant correlation between maximal length of rete ridge and PASI.

(a), (b), and (c): Lines represent linear regressions and  $R$ -values indicate the correlation coefficients of individual analyses. RFU, relative fluorescent intensity unit.

### **Figure 3. Cytokine production by iNKT cells in psoriasis plaque and length of rete ridge**

(a, b) Multicolour fluorescence staining of iNKT cells using (red) PE-labelled anti-TCRV $\alpha$ 24 antibody and (green) FITC-labelled anti-IFN- $\gamma$  antibody or FITC-labelled anti-IL-4 antibody in (a) PV and (b) AD sections.

- (c) Percentages of IFN- $\gamma$ - or IL-4+ iNKT cells in AD. \* $P=0.01$ , \*\* $P=0.02$  vs. PV.
- (d, e) Regression analyses with respect to the number per high power field (x 400) of iNKT cells positive for IFN- $\gamma$  or IL-4 vs. maximal rete ridge length (nm) (d) and microabscess area length (nm) (e). Lines represent linear regressions.  $R^2$  values for individual analyses are shown. Blue and red squares indicate the data plots of IFN- $\gamma$ + iNKT cells and IL-4+ iNKT cells, respectively.

**Figure 4. Cytokine or CCR5 expression by iNKT cells and CCL5 expression in the capillary veins of skin**

- (a) Immunostaining with antibodies against PE-labelled (red) TCRV $\alpha$ 24/FITC-labelled (green) CCR5 (left), PE-labelled TCRV $\alpha$ /FITC-labelled control IgG (upper right), and PE-labelled CCL5/FITC-labelled TCRV $\alpha$ 24 (lower right).
- (b) Immunostaining with antibodies against PE-labelled (red) CD31/FITC-labelled (green) CCL5.
- (c) Percentages of CCR5+ iNKT cells in psoriasis plaque and normal skin. \* $P<0.01$ , vs. AD and normal skin.
- (d) Counts per high power field (x 400) of PE-labelled CCL5+ capillary endothelial cells in psoriasis plaque and normal skin per 5 dermal papillae. \* $P<0.01$ , vs. AD and normal skin.
- (e, f) A regression analysis with respect to the number of PE-labelled CCL5+ capillary endothelial cells in psoriasis plaque per high power field (x 400) and rete ridge length (e) or microabscess area length (f).

**Figure 5.** A scheme of interaction of IFN- $\gamma$ + or CCR5+ iNKT cells with CCL5+ capillary veins in the psoriasiform hyperplasia of PV.

## Tables

**Table 1. PV patient profiles.**

S.E., somatic erythematous desquamation.

**Table 2. AD patient profiles**

S.E., somatic erythematous desquamation.

+ evident in the whole epidermis; ± localized; - negative

**TABLE 1. PV patient profiles**

Patient	Sex	Age (yr)	Biopsied loci	Rete ridge (min-max: µm)	Micro-abscess	Lymphocytic infiltrates	Proliferation of capillaries	Parakeratosis	Dyskeratosis	Scaling	Granular layer loss	Duration of disease	Other Clinical findings	PASI
1	M	40-50	abdominal	112-361	+	mild	++	+	+	+	+	1 year	S.E.	4.2
2	M	20-30	back	61-552	+	mild	+	++	+	+	+	5 years	S.E.	3.3
3	M	60-70	knee	101-302	+	mild	+	+	+	+	+	1 year	S.E.	6.5
4	F	40-50	leg	52-408	+	mild	+	+	+	+	+	20 years	S.E.	3.7
5	F	50-60	abdominal	81-251	+	moderate	+	+	+	+	+	4-5 years	S.E.	6.1
6	M	60-70	chest	70-503	+	moderate	+	+	+	+	+	6-7 years	S.E.	4.5

7	F	70-80	abdominal	100-196	+	moderate	+	+	+	+	+	2-3 months	S.E.	5.1
8	M	60-70	buttock	62-351	+	moderate	+	+	+	+	+	6 months	S.E.	3.3
9	M	40-50	leg	70-463	+	mild	+	+	+	+	+	1 year	S.E.	2.4
10	M	30-40	abdominal	62-380	-	mild	+	++	+	+	+	2-3 years	keratotic erythema on the hand	3.7
11	M	60-70	leg	94-511	+	mild	+	+	+	+	+	3 years	S.E.	4.2
12	M	70-80	abdominal	70-248	-	moderate	+	+	+	+	+	6 years	S.E.	5.1
13	M	60-70	upper arm	93-203	+	mild	+	+	+	+	+	2 months	S.E.	5.3
14	M	60-70	leg	41-461	-	mild	++	+	+	+	+	12 years	S.E.	5.1
15	M	30-40	back	84-359	+	moderate	+	+	+	+	+	20 years	S.E.	3.9
16	M	40-50	left auricle	42-490	+	moderate	+	+	+	+	+	10 years	arthropathy	3.9
17	F	50-60	forearm	50-218	++	mild	+/-	+	+	+	+	10 years	arthropathy	4.5

**PASI: Psoriasis Area and Severity Index; S.E., Somatic Erythematous Desquamation.**

**TABLE 2. Patient profiles of atopic dermatitis**

Patient	Sex	Age (yr)	Biopsied loci	Lymphocytic infiltrates	Proliferation of capillaries	Parakeratosis	Dyskeratosis	Scaling	Granular layer loss	Duration of disease	Clinical findings
1	F	30-40	forearm	scant	++	±	-	+	-	20 years	Erythroderma
2	F	20-30	upper arm	mild	+	±	-	+	-	20 years	Somatic erythema
3	F	30-40	lumbar region	moderate	+	-	-	+	-	30 years	Red purpura on buttock and back
4	M	30-40	forearm	mild	+	±	-	+	-	30 years	Erythroderma
5	M	30-40	upper arm	mild	+	±	-	+	-	20 years	Erythroderma
6	M	30-40	forearm	mild	+	-	-	+	-	30 years	Erythroderma
7	F	30-40	forearm	scant	-	±	-	+	-	20 years	Erythroderma
8	F	20-30	upper arm	mild	+	±	-	+	-	30 years	Somatic erythema
9	F	30-40	forearm	moderate	+	-	-	+	-	30 years	Erythroderma
10	M	30-40	forearm	mild	+	±	-	+	-	20 years	Erythroderma

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11	M	30-40	upper arm	mild	+	±	-	+	-	10 years	Erythroderma
12	M	30-40	forearm	mild	+	-	-	+	-	20 years	Erythroderma
13	F	30-40	forearm	mild	++	±	-	+	-	20 years	Erythroderma
14	F	20-30	upper arm	mild	+	±	-	+	-	20 years	Somatic erythema
15	F	30-40	lumbar region	moderate	+	-	-	+	-	30 years	Erythroderma
16	M	30-40	forearm	mild	+	±	-	+	-	30 years	Erythroderma
17	M	30-40	upper arm	mild	+	±	-	+	-	20 years	Erythroderma

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S.E., somatic erythematous desquamation.

+ evident in the whole epidermis; ± localized; - negative

Figure 1

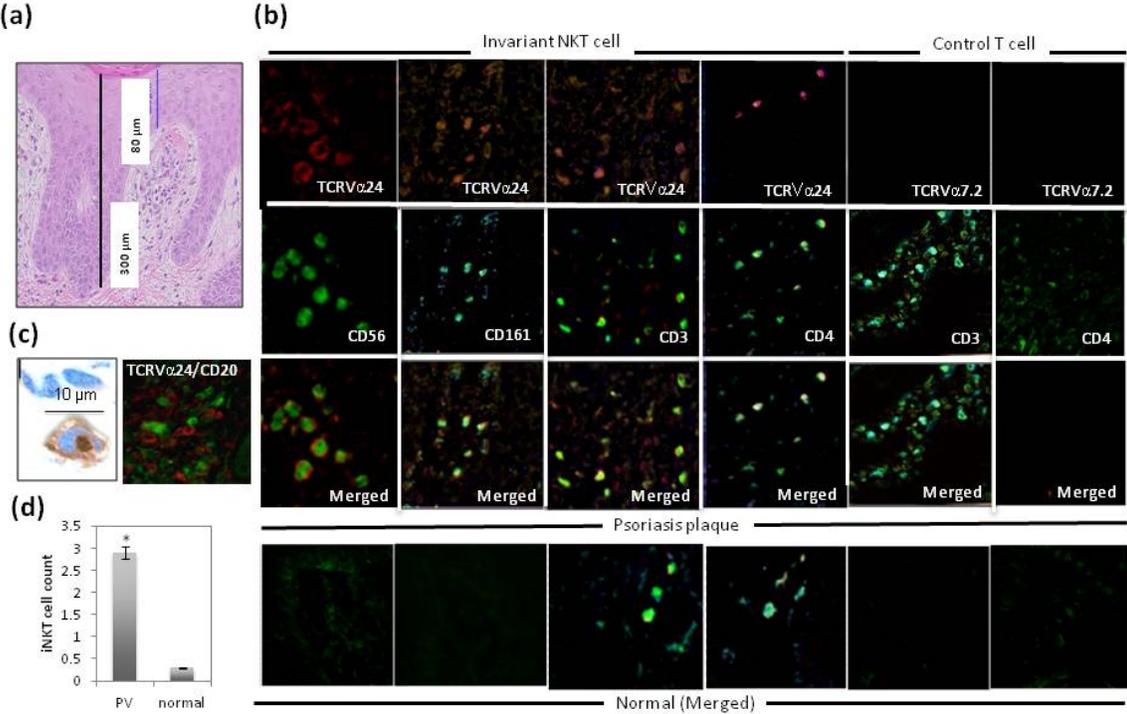


Figure 2

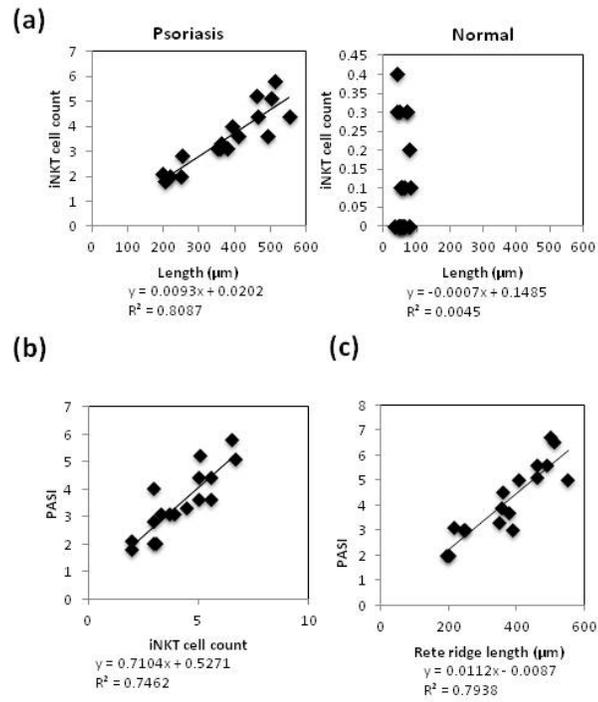


Figure 3

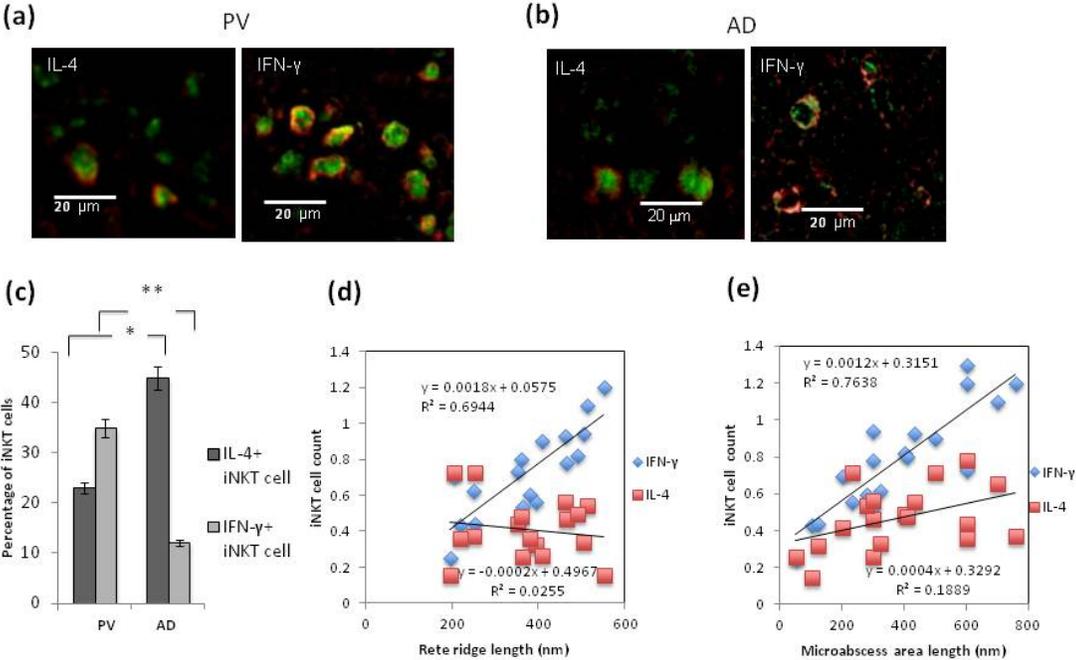


Figure 4

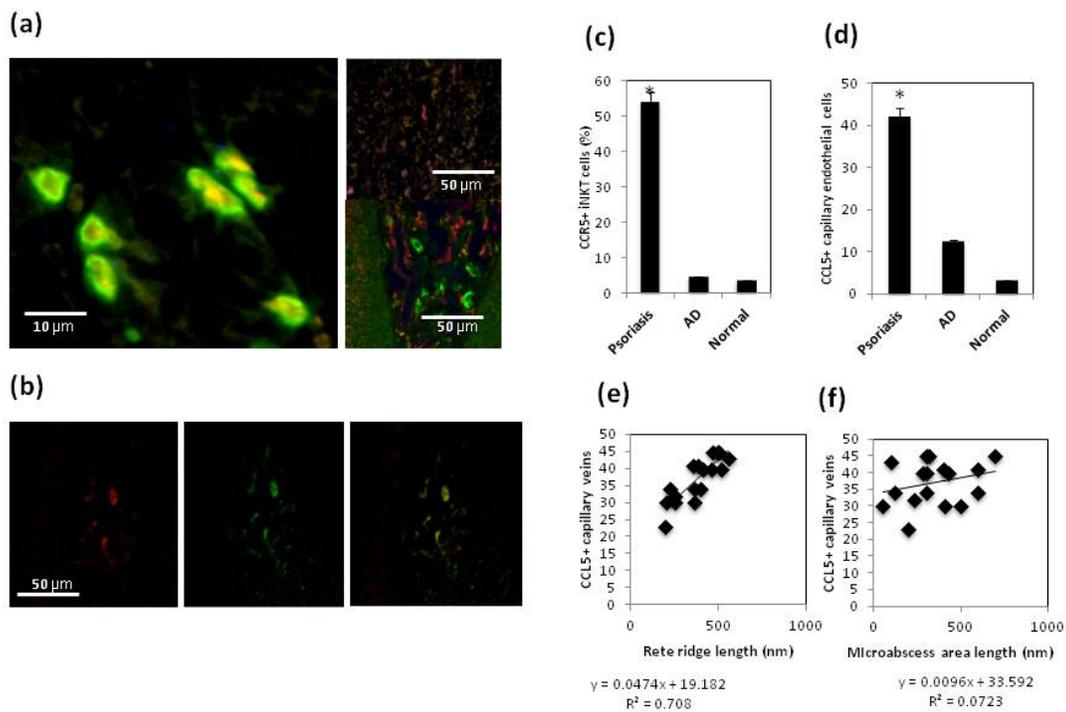


Figure 5

