New insight in vitiligo: immunopathology

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LOCAL IMMUNE RESPONSE IN SKIN OF GENERALIZED VITILIGO PATIENTS

DESTRUCTION OF MELANOCYTES IS ASSOCIATED WITH THE PROMINENT PRESENCE OF CLA+ T CELLS AT THE PERILESIONAL SITE

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Abstract

In situ immune infiltrates in lesional, perilesional and nonlesional skin biopsies from patients with vitiligo were analysed by immunohistochemical method as compared to those found in the skin of normal healthy donors and relevant selected disease controls. Increased influx of activated skin homing T cells and macrophages are seen in the perilesional biopsies. The overall percentages of CLA+ T cells were similar to those found in normal healthy donors, which is compatible with similar expression of E-selectin. Most strikingly, however the CLA+ T cells in perilesional skin were mainly clustered in the vicinity of disappearing melanocytes and 60 to 66% of these interacting T cells expressed perforin and granzyme-B. The perforin+/granzyme-B+ cells were not seen in locations other than that of disappearing melanocytes. Interestingly, the majority of the infiltrating T cells were HLA-DR+/CD8+. Another hallmark is the focal expression of ICAM-1 and HLA-DR in the epidermis at the site of interaction between the immune infiltrates and the disappearing melanocytes.

The data presented in this study are consistent with a major role for skin homing T cells in causing death of melanocytes as seen in vitiligo.

Introduction

Vitiligo is a cutaneous pigmentary disorder that affects approximately 1% of the world population1. It is characterised by milk-white macules of the skin and complete disappearance of melanocytes at the lesional site2. The etiology of melanocyte destruction in vitiligo remains largely unknown. The causal mechanism may vary for different patients3 4. However, an autoimmune mechanism has been put forward as an underlying cause. Such hypothesis was based on the observation that several autoimmune diseases are associated with vitiligo. In addition, increased autoantibody titers against melanocytic antigens have been reported5, and elevated serum levels of soluble IL-2-receptor correlating with disease activity were also reported in literature6. Particularly the latter parameter, observed in various infectious and auto-immune diseases, is related to the activation of immuno-competent cells7 8.

The auto-immune hypothesis gains further support from immunotherapy studies on melanoma patients9. Twenty-six percent of melanoma patients responding to IL-2 based immunotherapy developed vitiligo. This was in marked contrast to non responding melanoma patients and to the patient group with renal cell carcinoma where no vitiligo cases were seen. These findings suggest that anti-melanotic T cells responsible for melanoma regression may also
be linked to the destruction of normal melanocytes as seen in vitiligo and halo nevi. Indeed, it was clearly demonstrated that cytotoxic T cells (CTL) generated from melanoma tissue also recognise differentiation antigens expressed by normal melanocytes. It is well known that Melan-A is one of the melanocyte specific differentiation antigens often recognised by CTL in melanoma. In this respect, it is relevant that high frequency of skin-homing, Melan-A specific CD8+ T cells can be detected in peripheral blood of vitiligo patients. More recently it was further emphasised that some of these melanoma specific CTL that recognize melanocytic antigens also express the cutaneous leukocyte associated antigen (CLA); that is associated with homing to skin. Thus the relation between melanoma therapy and vitiligo is not surprising.

Inspite of this evidence, the in situ data on the role of infiltrating immunocompetent cells in generalized vitiligenous lesions are limited. We have however, reported that skin infiltrates of T cells and macrophages around the perilesional site parallel the development of lesions in a centrifugal manner in a rare type of depigmentary disease known as inflammatory vitiligo. Although in two of these studies, expression of CLA was evaluated, the importance of CLA+ T cell subsets in relation to melanocyte destruction was not explicitly demonstrated by immunodoublestaining method. Subsequently, in spite of these reports, it is still debated about to what extent local immune response is associated with the development of vitiligo. Such continued debate is due to the lack of elaborate in vivo studies on immunopathology of generalized vitiligo.

This prompted us to investigate further the in situ immuno-infiltrates in the nonlesional, perilesional and lesional skin of generalized vitiligo patients. In the present study, carefully selected biopsy materials were used for phenotypic characterisation of immunocompetent cells by immunohistochemical single and double stainings. The results clearly demonstrate, the in situ involvement of CLA+/CD8+ T cells in the destruction of melanocytes in generalized vitiligo.

Materials and methods

Tissue specimens

Ten patients diagnosed with generalized type of vitiligo were included in this study. Their demographic data are given in table 1. All patients had progressive disease, as evaluated by the patients own observations.
Table 1. Demographic data on the generalized type of vitiligo patients used in this study

<table>
<thead>
<tr>
<th>patient number</th>
<th>sex</th>
<th>age</th>
<th>biopsie from</th>
<th>duration vitiligo</th>
<th>course of lesion</th>
<th>medication</th>
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<tbody>
<tr>
<td>1*</td>
<td>M</td>
<td>54</td>
<td>arm</td>
<td>9 years</td>
<td>progressive</td>
<td>none</td>
</tr>
<tr>
<td>2*</td>
<td>F</td>
<td>49</td>
<td>leg</td>
<td>10 years</td>
<td>progressive</td>
<td>cellozane</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(blood pressure)</td>
</tr>
<tr>
<td>3*</td>
<td>M</td>
<td>43</td>
<td>arm</td>
<td>10 years</td>
<td>progressive</td>
<td>none</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>35</td>
<td>back</td>
<td>30 years</td>
<td>progressive</td>
<td>tetracyclen</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(acne)</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>38</td>
<td>hip</td>
<td>1 year</td>
<td>progressive</td>
<td>none</td>
</tr>
<tr>
<td>6*</td>
<td>F</td>
<td>33</td>
<td>hip</td>
<td>7 years</td>
<td>progressive</td>
<td>diprosone</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>40</td>
<td>arm</td>
<td>1 year</td>
<td>progressive</td>
<td>none</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>48</td>
<td>arm</td>
<td>4 months</td>
<td>progressive</td>
<td>none</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>26</td>
<td>foot</td>
<td>10 years</td>
<td>progressive</td>
<td>none</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>35</td>
<td>hip</td>
<td>5 years</td>
<td>progressive</td>
<td>none</td>
</tr>
</tbody>
</table>

* patient receiving psoralen/UVA (PUVA) treatment when biopsies were taken.

Four mm punch biopsies were taken from expanding lesions, snap frozen in liquid nitrogen and stored at −80 °C. Perilesional (PL) biopsies were taken after marking carefully the pigmented side bordering the vitiligenous spot with ink. Such procedure facilitated precise cutting of sections that span both lesonal (L) and nonlesional (NL) skin. From one of the 10 investigated patients only this PL skin was obtained. NL and L skin was taken 2 to 5 centimeters away from the PL area. As normal controls (NC), adult breast skin and abdominal skin specimens were obtained from plastic surgery tissue (n=4). Additional controls were obtained from the inside border of active psoriasis lesions (n=4) and patch tested (72 hours) delayed type hypersensitivity reactive skin from contact dermatitis patients (n=4). Six mm cryostat sections were cut, air dried, fixed in 4 °C acetone and stored at −20 °C until use.
Immunochemical single stainings

Cluster of differentiation codes (CD code), specificities, sources and working dilutions for all monoclonal antibodies (MoAbs) are given in table 2. Single staining procedures were performed according to the streptavidin-biotin immunoperoxidase method described earlier. Briefly, sections were preincubated in PBS plus 0.1% sodium-azide and 0.3% hydrogen-peroxide to avoid background staining by intracellular peroxidase. Following pre-incubation with 10% normal goat serum (Dakopatts), sections were incubated with primary MoAb's, using pre-evaluated working dilutions. This was followed by incubation with biotinylated rabbit anti mouse serum (1:200, Dakopatts), and next with strept ABC-Horse-Redish- Peroxidase complex (Dakopatts). Peroxidase activity was then visualized by 3-amino-9-ethyl carbazole (AEC, Sigma, St Louis, USA). In order to ensure the specificity, stainings were performed using isotype matched irrelevant monoclonal antibodies with specificity towards Aspergillus niger glucose oxidase (Dakopatts) instead of primary antibodies. In case of granzyme B stainings, sections were pre-treated by non-enzymatic antigen retrievel as was described earlier. Shortly, preparations were subjected to formalin fixation (4% buffered formalin, 10 minutes) and subsequent boiling in sodium citrate buffer (10 mM, PH 6.0, 10 minutes). Thereafter, sections were processed as described above.

Immunochemical double stainings

The following combinations of antibodies were used in immunohistochemical double staining procedures: CD3*FITC/CD8, CD3*FITC/HLA-DR, CD3*FITC/HECA-452, CD8*FITC/HECA-452, CD1a*FITC/CD68, CD8*FITC/NKI-Beteb, HLA-DR*FITC/NKI-Beteb, NKI-Beteb/CD68, NKI-Beteb/CD36.

Two different double staining methods were employed:

1) unlabeled MoAb was used first, followed by incubation with alkaline phosphatase labelled goat anti-mouse serum (1:20, Dakopatts). Subsequently, sections were incubated with the second MoAb which was FITC labelled, followed by rabbit anti-FITC (1:1000, Dakopatts) and peroxidase labelled swine anti rabbit (1:100, Dakopatts). Alkaline phosphatase activity was then visualised by Fast Blue BB (Sigma) and peroxidase activity by AEC.

2) In the NKI-Beteb/CD68 and NKI-Beteb/CD36 double stainings sections were first incu-bated with a mixture of the two MoAb's of different isotypes (NKI-Beteb:IgG2b, CD68, CD36:IgG1). In the next step two subclass specific polyclonal antibodies were mixed: alkaline phosphatase labelled goat-anti-mouse-IgG1 was used in a 1:10 dilution and peroxidase labelled goat-anti-mouse-IgG2b was diluted 1:20 (Southern Biotechnology, Birmingham, USA). Enzyme activities were then visualised with Fast Blue BB and AEC.
### Table 2. CD code, specificity, source and working dilution of all MoAbs used in this investigation

<table>
<thead>
<tr>
<th>MoAb/Source</th>
<th>CD code</th>
<th>Specificity</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT-6¹</td>
<td>CD1a</td>
<td>Langerhans cells</td>
<td>1:50</td>
</tr>
<tr>
<td>OKT-6*FITC¹</td>
<td>CD1a</td>
<td>Langerhans cells</td>
<td>1:100</td>
</tr>
<tr>
<td>Leu4²</td>
<td>CD3</td>
<td>All T cells</td>
<td>1:20</td>
</tr>
<tr>
<td>Leu4*FITC²</td>
<td>CD3</td>
<td>All T cells</td>
<td>1:200</td>
</tr>
<tr>
<td>Leu3a³</td>
<td>CD4</td>
<td>Helper T cells, subpopulation monocytes and macrophages</td>
<td>1:20</td>
</tr>
<tr>
<td>T8³</td>
<td>CD8</td>
<td>Suppressor and cytotoxic T cells</td>
<td>1:200</td>
</tr>
<tr>
<td>T8*FITC³</td>
<td>CD8</td>
<td>Suppressor and cytotoxic T cells</td>
<td>1:50</td>
</tr>
<tr>
<td>CD11a³</td>
<td>CD11a</td>
<td>Majority lymphoid and myeloid cells (LFA-1)</td>
<td>1:10</td>
</tr>
<tr>
<td>HD37³</td>
<td>CD19</td>
<td>All B cells</td>
<td>1:100</td>
</tr>
<tr>
<td>Leu14²</td>
<td>CD22</td>
<td>Precursor and mature B cells</td>
<td>1:1</td>
</tr>
<tr>
<td>Tu69⁵</td>
<td>CD25</td>
<td>Activated T and B cells Activation of T and B cells</td>
<td>1:100</td>
</tr>
<tr>
<td>OKM 5¹</td>
<td>CD36</td>
<td>Subpop. monocytes and macrophages</td>
<td>1:50</td>
</tr>
<tr>
<td>UCHL1³</td>
<td>CD45RO</td>
<td>Memory T cells</td>
<td>1:25</td>
</tr>
<tr>
<td>Leu18²</td>
<td>CD45RA</td>
<td>Virgin T cells</td>
<td>1:25</td>
</tr>
<tr>
<td>RR1⁶</td>
<td>CD54</td>
<td>ICAM-1 (broad tissue distribution)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Leu7²</td>
<td>CD57</td>
<td>Subpopulation of NK cells</td>
<td>1:20</td>
</tr>
<tr>
<td>BBIG-E4⁴</td>
<td>CD62E</td>
<td>Activated endothelial cells</td>
<td>1:100</td>
</tr>
<tr>
<td>KP1³</td>
<td>CD68</td>
<td>All macrophages</td>
<td>1:2000</td>
</tr>
<tr>
<td>UB2⁸</td>
<td>CD95</td>
<td>Fas (broad distribution)</td>
<td>1:100</td>
</tr>
<tr>
<td>33⁹</td>
<td>CD95-L</td>
<td>Fas-Ligand (broad distribution)</td>
<td>1:100</td>
</tr>
<tr>
<td>Delta G⁹¹⁰</td>
<td>perforin</td>
<td>Activated CTL and NK cells</td>
<td>1:100</td>
</tr>
<tr>
<td>GrB-7¹¹</td>
<td>Granzyme B</td>
<td>Activated CTL and NK cells</td>
<td>1:50</td>
</tr>
<tr>
<td>L243²</td>
<td>HLA-DR</td>
<td>MHC-II (HLA-DR specificity)</td>
<td>1:50</td>
</tr>
<tr>
<td>L243*FITC²</td>
<td>HLA-DR</td>
<td>MHC-II (HLA-DR specificity)</td>
<td>1:50</td>
</tr>
<tr>
<td>HECA-452¹²</td>
<td>CLA</td>
<td>Cutaneous lymphocyte-associated Antigen</td>
<td>1:50</td>
</tr>
<tr>
<td>NKI-Beteb¹¹</td>
<td>GP-100</td>
<td>Melanocyte differentiation antigen</td>
<td>1:40</td>
</tr>
</tbody>
</table>
Evaluation of immunostaining

Since melanocytes are located in the basal layer of the epidermis, T cells in juxtaposition to melanocytes are located in the epidermis as well as in the papillary dermis. Therefore, in this investigation, the epidermis and the papillary dermis were evaluated as one compartment, the number of stained cells was quantified per mm of epidermis.

The relative distribution of CD4+ and CD8+ T cells in the infiltrates was evaluated using CD3/CD8 double stainings. CD4+ T cells were counted as the total number of CD3+ cells minus the number of CD3/CD8 double positive cells. In the case of CD68 and CD1a, positive cell numbers were too abundant for accurate counting. Stained cells were quantified using an arbitrary scale ranging from 1+ to 3+. Percentages of HECA-452/CD3 and HLA-DR/CD3 double positive cells were scored by evaluating clusters of infiltrating cells in 5 separate fields and then calculating the average percentage as well as by counting dispersely located double positive cells. Stainings were evaluated by two independent observers.

Differences between normal control and patient results were tested for statistical significance using nonparametric Mann-Whitney test and are considered significant when p values are less than 0.05.

Results

Melanocytes

Similar melanocyte distribution patterns were found in NC and NL vitiligo skin whereas melanocytes were absent from the lesional part of the PL epidermis (figure 1a). At the true border of the PL site remnants of disappearing melanocytes could be seen, and such melanocyte fragments were not observed in NL skin. Importantly, the observation of melanocyte degeneration in perilesional skin confirms our earlier findings obtained by confocal laser scanning microscopy. The use of this technique ruled out the possibility that melanocyte fragmentation is observed due to the fact that dendritic processes of a melanocyte could be in another field of view that might mask the entire cell body. L skin of all patients was totally depleted of NKI-Beteb positive melanocytes.
Immuo-doublestaining revealed that 30% of melanocytes (median value) in PL skin sections of the vitiligo patients expressed HLA-DR molecules. Double positive melanocytes were particularly seen in parallel with cellular infiltrates. A higher percentage of HLA-DR/NKI-beteb double positive cells was observed in PL sections when a relatively low number of remaining melanocytes was present (example in figure 2a). In NL skin such double positive cells (8%) were detected in only one patient, whereas normal controls were always negative. In this context, in other disease control specimens such as psoriasis and contact dermatitis, low percentage HLA-DR positive melanocytes are occasionally seen (data not shown). Further, melanocytes in all the investigated samples expressed CD68 antigen with an intensity comparable to that of dermal macrophages (see macrophage section). An example of NKI-Beteb/CD68 double staining is shown in figure 2b.
**T cells**

An example of the distribution pattern of CD3\(^+\) T cells in PL vitiligo skin is presented in figure 1b. T cells were focally present in the epidermal/papillary area and were mainly located around the basal epidermal layer. In the reticular dermis both perivascularly located and scattered CD3\(^+\) T cells can be observed. In table 3 CD4/CD8 ratios are shown. It can be observed that CD8\(^+\) T cells compose the major part of T cell infiltrates in generalized vitiligo skin.

<table>
<thead>
<tr>
<th></th>
<th>nonlesional</th>
<th>perilesional</th>
<th>lesional</th>
<th>normal control</th>
</tr>
</thead>
<tbody>
<tr>
<td>epidermis &amp; papillary dermis</td>
<td>0.4 (±0.3)</td>
<td>0.4 (±0.3)</td>
<td>0.6 (±0.9)</td>
<td>1.1 (±0.8)</td>
</tr>
<tr>
<td>reticular dermis</td>
<td>0.3 (±0.2)</td>
<td>0.5 (±0.4)</td>
<td>0.4 (±0.3)</td>
<td>1.5 (±0.3)</td>
</tr>
</tbody>
</table>

CD4/CD8 ratios (mean ± SD) in generalized vitiligo (n=7) and normal control skin (n=3) as determined from CD3/CD8 immunohistochemical double stainings (see the text).

Total numbers of infiltrating CD8\(^+\) T cells are given in figure 3. In spite of marked donor to donor variation, the highest numbers of CD8\(^+\) T cells were found in the PL area. In the dermal compartment as well as in the epidermis/papillary dermis of PL skin, where the injured melanocytes are seen, the number of CD8\(^+\) T cells was significantly higher (p<0.005 versus number of positive cells in NC skin). CD8\(^+\) T cells apposed to remaining PL melanocytes and melanocytic remnants can be clearly seen in NKI-Beteb/CD8 double stainings, an example of which is shown in figure 2c. Incidentally, such T cell/melanocyte interactions were also observed in psoriasis and contact dermatitis skin sections but without degeneration of melanocytes (data not shown).

The number of infiltrating T cells is elevated in PL vitiligo skin, however, the percentage of cutaneous lymphocyte associated antigen (CLA) expressing T cells is not increased and compares to that observed in NC and contact dermatitis skin (40-60%). On the other hand 80% of the infiltrating T cells in psoriasis were CLA\(^+\). Serial sections show that in vitiligo PL skin CLA\(^+\) T cells are primarily clustered at the epidermal/dermal junction at the site of melanocyte destruction (figures 2c and 2d). In order to ascertain whether these T cells may have the ability to participate in cytotoxicity we performed granzyme-B and perforin stainings. Indeed, granzyme-B and perforin positivity was detected in PL skin exactly at the spots where T cells interacted with melanocytes (see serial sections in figures 2c, 2d, 2e and 2f) and was never observed in either
NL, L or NC skin. To evaluate how many of these interacting T cells were granzyme-B and perforin positive, serial sections stained for NKI-Beteb/CD8, NKI-beteb/CD3 (double stainings) and perforin and granzyme-B (single stainings) were counted. It was found that most of the T cells in apposition to melanocytes were CD8+ (average CD4/CD8 ratio 0.48). Most importantly, 66% "14% (SD) of these interacting T cells expressed granzyme-B and 60% "34% (SD) expressed perforin. Further, sections were also stained for Fas and Fas-ligand. Unfortunately, due to staining of the whole epidermis for both molecules in all specimens, accurate evaluation of the Fas and Fas-ligand expression by interacting T cells and melanocytes at the epidermal/dermal junction was not possible (data not shown).

Infiltrating T cells were mostly of the CD45RO memory type and also express LFA-1 (data not shown). Figure 4 illustrates the number of CD25 expressing cells which is highest in PL vitiligo skin (p<0.05 as compared to NC skin). Increased numbers of activated T cells in PL skin were also found by CD3/HLA-DR double stainings. In clusters of perivascularly located infiltrating cells, up to 70% of T cells were HLA-DR positive whereas in NC, NL and L skin percentages varied from 10 to 40%.

Macrophages:

Increased numbers of macrophages, as determined by pan-macrophage marker, were found in nine out of ten patients when compared to NC skin. More CD68+ cells were found particularly in the papillary dermis of PL and L skin. These data are summarized in table 4. An example of this staining pattern in PL vitiligo skin as compared to NL skin is shown in figures 5a and 5b. Moreover, CD1a/CD68 double stainings revealed that a subset of epidermal Langerhans cells in vitiligo as well as normal control skin sections express the CD68 antigen (results not shown). Using anti CD68 monoclonal antibody at a forty fold increased concentration we observed that the antibody is reactive with all epidermal keratinocytes. This expression pattern was apparent in NC and NL vitiligo skin and was markedly reduced in L vitiligo skin. An example is shown in figure 5c where a section spanning both L and NL skin (according to serial NKI-Beteb staining) is shown.

Melanocytes expressed CD36, which is also known as a macrophage marker. CD36 staining intensity of melanocytes varied, even for melanocytes within one section. Normal control as well as NL and PL vitiligo melanocytes were found reactive. Consequently, the total number of CD36+ macrophages in each section was evaluated by substracting the number of NKI-Beteb/CD36 double positive cells from the total number of CD36+ cells (figure 6). The observed differences were not statistically significant according to Mann-Whitney test.
immune infiltrates in progressive vitiligo

Figure 2 (A). NKI-Beteb(blue)/HLA-DR(red) double staining showing HLA-DR positivity of one remaining melanocyte (arrow) in PL skin. (B) NKI-Beteb(red)/CD68(blue) double staining of normal control skin showing CD68+ melanocytes (arrows). Magnifications: A, x132, B, x100

Figure 2(C, D, E, F). Serial sections of PL vitiligo skin; (C) NKI-Beteb(blue)/CD8(red) double staining, long arrow shows; CD8+ T cell apposed to melanocyte, short arrows show; CD8+ T cells apposed to melanocytic remnants. (D) CD3(red)/CLA(blue) double staining showing cluster of double positive T cells (arrows) at the perilesional epidermal/dermal junction and CD3 single stained cells in the dermal compartment (asterisks). (E) Granzyme-B and (F) perforin positive cells at the perilesional epidermal/dermal junction. Magnifications: C, x132, D, E and F x 160.

Color reproduction of Figure 2 A-D in last section of this thesis.
**CD8**$^+$ T cells

![Diagram of CD8$^+$ T cells](image)

*Figure 3: Number of CD8$^+$ T-cells/mm epidermal length (mean +/- SD) as calculated from CD8 single stainings. * P value < 0.005 versus normal control skin.

**CD25**$^+$ cells

![Diagram of CD25$^+$ cells](image)

*Figure 4: Number of CD25$^+$ cells/mm epidermal length (mean +/- SD) as calculated from CD25 stainings. ** P value < 0.05 versus normal control skin.

**B Cells and NK Cells:**

No B cells and no NK cells were detected in vitiligo and control skin.

**Langerhans cells:**

Considerable donor-to-donor variation was observed in epidermal Langerhans cell numbers both within the generalized vitiligo as well as within the normal control group. In one of the ten investigated patients more Langerhans cells were detected in PL epidermis when compared to L or NL skin. Decreased Langerhans cell numbers were observed in L skin of seven patients, four of which were receiving ultraviolet-A radiation (UVA) therapy. Additionally, there was a tendency of increased numbers of CD1a positive cells located along the basement membrane in PL and especially in L skin in all investigated patients. A representative example of such basal localisation in PL skin is given in figure 7.
immune infiltrates in progressive vitiligo

Table 4. CD68+ macrophages in papilary dermis of vitiligo and normal control skin

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<tr>
<th>patient number</th>
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<th>perilesional skin</th>
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<td>1+</td>
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<td>1+</td>
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<td>normal donor 3</td>
<td>1+</td>
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<td>2+</td>
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Keratinocytes:
Focal expression of HLA-DR, coinciding with the cellular infiltrates at the site of melanocyte loss, was detected in PL epidermis of eight out of ten patients.

Relevant cell adhesion molecules (E-selectin & ICAM-1):
Vascular expression of E-selectin was found in all patient samples as well as control specimens. When compared to normal control tissue, E-selectin expression did not appear to be upregulated, nor expressed in a higher number of post-capillary venules in vitiligo skin. In contrast to this, the number of E-selectin positive post-capillary venules in psoriasis skin was significantly higher (p<0.05) than in normal control skin (figure 8). Importantly, despite the lack of E-selectin elevation in vitiligo skin over normal controls, T cell infiltration, when observed, was restricted to those venules that expressed E-selectin.
ICAM-1 was detected on capillaries in the reticular and papillary dermis as well as on perivascularly located and scattered infiltrating cells. Focal epidermal expression was found in PL skin of eight out of ten patients (figure 9), in contrast to NC or NL and L vitiligo skin. The observed epidermal ICAM-1 expression coincided with the expression of HLA-DR according to staining of serial sections.

Figure 5 (A). Anti-CD68 immuno-enzymatic staining of nonlesional and (B) perilesional skin showing macrophage infiltrates.

Figure 5 (C). Epidermal anti-CD68 expression pattern in perilesional skin using high antibody concentration. Magnifications: A and B, x66; C, x16.
CD36$^+$ cells

Figure 6. Number of CD36$^+$ cells/mm epidermal length (mean +/- SD) as calculated from NKI-Beteb double stainings.

E-selectin$^+$ post-capillary venules

Figure 8. Number of E-selectin$^+$ capillaries/mm epidermal length (mean +/- SD) as calculated from E-selectin single stainings.

* * P value < 0.05 vs normal control skin.

Figure 7. Basal localisation of CD1a reactive Langerhans cells in the lesional epidermis of a vitiligo donor (magnification: x50).

Figure 9. ICAM-1 reactivity in perilesional skin of vitiligo donor. Note the focal epidermal expression Indicated by the arrow (magnification: x50).
Discussion

The present study showing the consistent presence of immune-infiltrates in perilesional skin of patients with the generalized type of vitiligo, confirms that an ongoing local immune response is associated with melanocyte destruction. The immune infiltrates are mainly composed of T cells and macrophages. The number of infiltrating T cells, consisting of mostly CD8^+CD45RO^+ cells, was highest in the perilesional area. These T cells were frequently found in apposition to melanocytes and melanocyte remnants along the basement membrane and were in activated state, as evident from their HLA-DR expressions. Moreover, some of these T cells with the expression of CD25 were seen in increased amount in perilesional skin. This indicates that ongoing T cell activation is involved in the progressive depigmentation process. Such assumption gains further support from the observation that the majority of T cells that was detected in juxtaposition to perilesional melanocytes was perforin and granzyme-B positive. Since, these effector molecules of lymphocyte granule-mediated apoptosis were never observed in NL, L or NC skin specimens their apparent apposition to perilesional melanocytes is not, therefore, coincidental. Interestingly, with respect to the other well established pathway for CTL induced apoptosis such as Fas and Fas-ligand, our unpublished in vitro observations clearly indicate that melanocytes are insensitive to Fas-ligand mediated cell death (van den Wijngaard et al, manuscript in preparation). This observation is compatible with the report that, inspite of melanocytic expression of Fas, Fas-ligand mediated apoptosis cannot be induced in these cells.

Further, the prevalence of CD8^+ T cells over CD4^+ T cells appears to be a prominent feature in generalized vitiligo. Such an imbalance in CD4/CD8 ratios was also reported in a study on the Smyth line chicken animal model for vitiligo, where CD8^+ T cells prevail following the onset of the depigmentation process. Furthermore, the presence of activated T cells in generalized vitiligo, may be due to the preferential migration of skin homing, CLA^+ T cells near target melanocytes. In this regard the present study demonstrated, that the relative number of CLA-expressing cells in perilesional infiltrates compares to the normal situation and other inflammatory skin disease such as contact dermatitis. The absence of a relative increase in CLA expressing cells is compatible with the normal number of E-selectin expressing post-capillary venules. Because E-selectin on vascular endothelium is the natural ligand for CLA^+ immune infiltrating cells. Interestingly, E-selectin expression does however colocalize with the perivascular T cell infiltration in perilesional skin and the absolute number of CLA^+ T cells is higher in perilesional vitiligo skin as compared to nonlesional skin. Importantly, CLA^+ T cells are frequently detected as clusters at the epidermal/dermal junction where melanocytes are in the
immune infiltrates in progressive vitiligo

process of dissappearing. In psoriasis and contact dermatitis, however, such CLA$^+$ T cells are incidently observed in apposition to melanocytes and melanocyte destruction does not occur in these inflammatory dermatoses. Therefore, it is likely that skin homing T cells in vitiligo perilesional skin are indeed specifically involved in melanocyte destruction.

In conjunction with the T cell infiltrates, we also observed HLA-DR expression by perilesional melanocytes. In this respect, it was previously suggested that melanocytic HLA-DR expression may contribute to the destruction of melanocytes in pigmentary disorders. Such assumption was based upon in vitro experiments where it was demonstrated that melanocytes can process antigen and present its antigenic peptides to HLA-DR restricted T cell clones$^{26}$. As these clones were able to respond both in a proliferative and a cytotoxic fashion it was concluded that HLA-DR expressing melanocytes can contribute to the local immune response in vivo by presenting antigen, and simultaneously, become targets for cytotoxic T cells. Besides focal expression of HLA-DR, concomittant expression of ICAM-1 in the epidermis indicates that this costimulatory molecule may render melanocytes susceptible to recruited antigen specific T cells.

In addition to T cell infiltrates, we also demonstrate macrophage infiltrates in vitiliginous skin. Macrophage infiltrates were characterized using the pan-macrophage marker CD68 as well as macrophage subset marker CD36. Macrophage CD36 expression was investigated because it has been described that this molecule promotes efficient phagocytosis of apoptotic cells$^{27}$. In addition, it is now well documented that cytotoxic T cells kill their target cells via the induction of apoptosis$^{20,21}$. Despite this, a significant increase of CD36 positive cells was not observed at the site of melanocyte disappearence. However, in nine out of ten patients increased numbers of CD68$^+$ macrophages were present in perilesional skin. In addition to the possibility that infiltrating macrophages are involved in clearing apoptotic cells$^{28}$, a direct involvement of macrophages in melanocyte killing, probably via nitric oxide (NO) pathway$^{29}$, cannot be ruled out. In this respect, using SIN-1 as a NO donor, it has been observed that NO induces detachment and apoptosis of melanocytes cultured on fibronectin coated plates (Ivanova, personal communication).

When investigating the distribution of the pan macrophage marker CD68, double stainings showed some Langerhans cells but all melanocytes to be CD68$^+$. The expression of CD68 by Langerhans cells is in agreement with earlier reports$^{30}$. Since CD68 and also CD36 are phagocytosis related markers, their expressions on melanocytes are compatible with the fact that pigment cells have phagocytic capacity$^{31}$. Furthermore, CD36 expression on melanocytic cells was also reported by others$^{32,33}$. In addition to epidermal expression of macrophage markers by melanocytes and Langerhans cells, we observed that the whole epidermis also shows CD68-
expression when using high antibody concentrations. This expression is high in skin sections where melanocytes are present (both in normal control and non lesional vitiligo skin) and reduced when melanocytes are absent. The latter finding is consistent among patients and compares with that reported for inflammatory vitiligo. The markedly reduced expression in melanocyte depleted lesional skin suggests that CD68 expression in control and non involved vitiligo epidermis may be due to melanocytes. This implies that the absence of intense CD68 reactivity is a feature of lesional skin in all hypopigmentary disorders. In this respect, we observed similar staining patterns in all of 3 patients with the congenital hypopigmentary disorder called piebaldism (not shown).

Despite the expression of different immunological markers by epidermal cells, the main focus of this study is the interaction of locally recruited immunocompetent cells in relation to vitiligo pathology. In this respect, another important cell is the epidermal Langerhans cell. We observed a decreased number of lesional Langerhans cells in seven out of ten patients. Four of these patients were receiving UVA therapy and it has been described that such therapy leads to a decreased presence of Langerhans cells in lesional vitiligo skin. Surprisingly, the tendency toward a more basal location of Langerhans cells as seen in perilesional and lesional skin was independant of UVA exposure, this is in agreement with the results reported in literature.

The presence of in situ immune infiltrates and their interactions with epidermal melanocytes are a major feature in vitiligo pathology. In spite of this, the absolute number of infiltrating cells in perilesional skin is limited as compared to other inflammatory skin diseases. This underscores the importance of careful selection of biopsy material in the sense that when perilesional biopsies do not span lesional and nonlesional skin of an expanding lesion it may be falsely concluded that immune infiltrates are not involved in vitiligo pathogenesis. In this respect, the presence of only limited numbers of infiltrating cells may be attributed to either 1) the low number and dispersed localization of target cells (melanocytes) or 2) T cells recirculate after delivering their lethal hits or 3) infiltrating T cells enter activation induced apoptosis. The latter possibility also underscores the importance of infiltrating macrophages as they efficiently clear apoptotic cells.

Recently it was also demonstrated that CLA expressing CTL, specific for the melanocyte differentiation antigen Melan-A can be isolated from peripheral blood of vitiligo patients. Importantly, blood derived Melan-A specific CTL derived from a normal control donor did not express CLA. Moreover, in cooperation with these investigators, we could demonstrate Melan-A specific, CLA+/CD8+ T cells in cell lines derived from perilesional vitiligo skin biopsies (Kalinska et al, manuscript in preparation). Together with the in situ data presented in this report,
this proves that CLA expression is an essential feature for effective autoreactive response by melanocyte specific T cells in PL skin. Since the present study clearly shows the increased in situ presence of activated, granzyme-B and perforin expressing, CLA+/CD8+ T cells, as well as their apposition to the disappearing melanocytes at the perilesional site we conclude, that in vivo destruction of melanocytes in vitiligo is primarily mediated by these skin homing CD8+ T cells.
References


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chapter 7

PROTECTIVE MECHANISMS IN LETHAL T CELL / MELANOCYTE INTERACTIONS

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