Melanocyte-specific Immune Response in a Patient with Multiple Regressing Nevi and a History of Melanoma

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Abstract. *Background/Aim: Regressing nevi are considered an example of an efficient early antitumoral response preventing the development of neoplasia. The underlying mechanism has not been elucidated, although an immune-based destruction of melanocytes is supposed. The aim of this study was to provide evidence of an effective immunosurveillance of pigment lesions in a patient at high risk of melanoma. Case Report: A patient with the dysplastic nevus syndrome and a history of melanoma was included in this study. Since 2003, a marked regression of almost all nevi was observed. Immunohistochemistry was performed and the antigen specificity of T-cells was analyzed on T-cells isolated from a regressing nevus by flow cytometry using HLA-A2-peptide tetramers containing Mart-1_{26-35}, $gp100_{280-288}$, $gp100_{209-217}$ and tyrosinase$_{369-377}$. Immunohistochemistry of the regressing nevi showed a strong infiltrate of CD4 + and CD8 + T-cells. Flow cytometric analyses demonstrated the presence of a CD8 + T-cell response against $gp100_{280-288}$ and Mart-1_{26-35} both in peripheral blood and in a regressing nevus. Conclusion: These findings indicate that an immune reaction against melanocyte differentiation antigens can target specifically nevi without signs of vitiligo and suggests that boosting the anti-melanocyte immune response in patients at high risk for melanoma may prevent tumor development at an early stage.*

The regression of benign and malignant pigment lesions is a well known phenomenon. Regressing nevi are a relatively frequent observation, occurring in approximately 1% of the population. Most commonly, a regressing nevus is accompanied by a surrounding white depigmented area, called a halo. Halo nevi are most frequently observed in adolescents with an average age of onset of 15 years (1). No obvious triggering factors are known. We have previously reported that stress (60%) and puberty (40%) were most commonly reported as triggering factors (2). UV exposure has been proposed as an eliciting factor, although this may reflect only an easier detection of the halo contrasting with tanned skin (3).

The phenomenon of regressing nevi without halo is not well documented in the literature. In daily practice, the regression of nevi without halo is, without use of good follow-up pictures, clinically difficult to observe. Most commonly, the diagnosis of a regressing nevus without halo is made by the histopathologist, mentioning a band-like lymphocytic infiltrate comparable to halo nevi. Therefore, the phenomenon of regressing nevi without halo has been termed 'nevi with halo-like reaction' (4). Epidemiological data about possible differences in age of occurrence or triggering factors are lacking.

The immunosurveillance of (dysplastic) melanocytic lesions is of major importance given the possibility to progress to melanoma. Lymphohistiocytic infiltrates are present in a large proportion of melanocytic nevi (78%) (5). The etiopathological mechanism of regressing nevi is still largely unknown. The marked inflammatory infiltrate seen in the histopathological examination suggests an immune-based mechanism. Nonetheless, clear evidence for a melanocyte-specific immune reaction is lacking. Musette *et al.* (6) detected in halo nevi a limited subset of T-cells which showed an oligoclonal expansion most likely resulting from specific antigen recognition. In patients with multiple halo nevi, the same type of oligoclonal T-cells were found in different halo nevi, implicating that the antigens are probably shared between these nevi. However, these oligoclonal T-cells could not be detected in the circulation (6).

Patients with dysplastic nevus syndrome are characterized by more than 100 nevi with one nevus more than 8 mm diameter and at least one atypical nevus. Individuals with

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atypical nevi have an approximately 15-fold higher risk of developing melanoma (7). A close follow-up of these patients is crucial given the importance of early detection on survival. More tumor-infiltrating lymphocytes were found in dysplastic nevi than in benign nevi. This may be explained by an increased expression of melanocyte differentiation antigens (MDAs) and an increased production of lymphocyte-recruiting chemokines and adhesion molecules. MDAs (*e.g.* Mart-1, gp100 and tyrosinase) are the largest known subset of peptide antigens that are reported to elicit immune responses in patients with vitiligo and melanoma. They are derived from proteins which reside in the melanosomes and are involved in pigment synthesis. MDAs are also expressed by melanoma cells and their expression is associated with an improved survival of stage IV melanoma patients (8). Therefore, patients with dysplastic nevus syndrome might have an increased likelihood of developing an immune reaction against melanocytic antigens (9).

Materials and Methods

Patients. The patients were recruited at the Department of Dermatology of the Ghent University Hospital and signed an informed consent. The study was approved by the Ethical Committee of the Ghent University Hospital, according to the Helsinki Principles.

Immunohistochemistry. The immunohistochemical stainings were carried out with the standard avidin-biotin complex (ABC) protocol on 4 μm-thick sections. 3-Amino-9-ethylcarbazole (AEC) was used as chromogen and nuclear counterstaining was performed with haematoxylin. The sections were incubated with CD3 (Dako, Heverlee, Belgium), CD4 (Dako), CD8 (Dako), CD11a (1/200; eBioscience, Hatfield, UK), forkhead box P3 (FoxP3) (1/100; eBioscience, San Diego, USA) and monoclonal anti-indoleamine 2,3 dioxygenase (clone 10.1, 1/200; Millipore, Billerica, USA) antibodies. For all stainings, an incubation time of 30 minutes was used, except for FoxP3 and IDO, which required an incubation time of 1 hour.

Peripheral blood mononuclear cell (PBMC) isolation. Peripheral venous blood was drawn in EDTA tubes, diluted 1:2 with RPMI 1640+ GlutaMAX™-I (Invitrogen, Merelbeke, Belgium) and PBMCs were separated by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) gradient centrifugation. The cells were washed three times and cryopreserved at –80˚C in fetal bovine serum (FBS) (Invitrogen, Merelbeke, Belgium) supplemented with 10% dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and 1% Pen Strep (Invitrogen, Merelbeke, Belgium) until analysis.

HLA typing. HLA typing was carried out by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human HLA-A2 specific antibody (HLA-A2 FITC; BD Biosciences, Breda, the Netherlands) and a biotinylated HLA-A2-specific antibody (BIH0648; OneLambda, Canoga Park, CA, USA) visualized by allophycocyanin (APC)-conjugated streptavidin. As negative controls, IgG1-FITC (BD Biosciences) and IgM-biotin isotype control antibodies (OneLambda) were used.

Isolation of skin lymphocytes of the regressing nevus. The isolation of skin lymphocytes was performed according to Clark *et al*. (10). Briefly, the skin was cut into pieces and cultured on grids in a medium containing Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FBS, 1% penicillin streptomycin (Pen Strep; Invitrogen), and 3.5 μl/l β-mercaptoethanol (Fluka, Biochemika, Buchs, Switzerland). T-cell expansion was performed over 2 weeks by adding anti-CD3 and anti-CD28 antibody-coated beads (Invitrogen).

Tetramer analysis. Flow cytometric analyses were performed with HLA-A2/peptide tetramers for the melanocyte differentiation antigens Mart-1, gp100, tyrosinase and a control antigen (influenza virus) on the lymphocytes isolated from the regressing nevus and the peripheral blood, as previously described (11). Phycoerythrin (PE)-conjugated HLA-A2/peptide complex tetramers containing melanocyte differentiation antigens Mart-1_{26-35} [modified position 27 (A>L): (ELAGIGILTV)], $gp100_{280-288}$ (YLEPGPVTA), $gp100_{209-217}$ (ITDQVPFSV), $tyrosinase_{369-377}$ (YMDGTMSQV), or control influenza $virus_{58-66}$ (GILGFVFTL) peptide were used. After incubation of the lymphocytes for 10 minutes with HLA-A2/peptide tetramers in phosphate-buffered saline with 1% bovine serum albumin at room temperature, the cells were stained with a FITC-conjugated anti-human CD8 antibody (CD8-FITC; BD Biosciences). Tetramer-binding to CD8 + T-cells was analyzed by flow cytometry using a FACS Canto II (BD Biosciences), acquiring approximately 300,000 events per sample. Data were analyzed with FLOWJO 7.6.2 software (Treestar Inc, San Carlos, USA).

Results

Patient with regressing nevi. A 59-year-old man with multiple dysplastic nevi (>100) had been in follow-up since 1997 at our Department. In 1999, he developed a superficial spreading melanoma (<1 mm) that was excised with a free margin of 2 cm. He was regularly followed-up at our Department and developed gradually regression of multiple nevi without evidence of a halo phenomenon (Figure 1). The patient took no medication except a course of isotretinoin which was prescribed for persisting acne vulgaris in 2003. In 2001, two pigment lesions were excised which showed an intradermal nevus and a dysplastic nevus without a clear inflammatory infiltrate. In contrast, an excision of a nevus in 2007 showed a marked inflammatory reaction corresponding to a halo phenomenon around a dysplastic nevus with cytological atypia. In 2010, a regressing nevus was removed for research, which showed also a dense, almost band-like lymphocytic T-cell infiltrate around nests of nevus cells consisting mainly of $CD3^+$, $CD4^+$, $CD8^+$ and $CD11a^+$ lymphocytes. An enhanced frequency of $CD1a^+$ Langerhans cells was present (Figure 2). A normal proportion of FoxP3+ cells was observed (5.7% of T-cells) and indoleamine 2,3-dioxygenase activity, a modifier of inflammation, was present.

Characteristics of T-cells and assessment of melanocyte antigen-specific T-cells in the blood and regressing nevi. To assess the T-cell response in regressing nevi, we isolated skin

Table I. *Tetramer analysis of melanocyte differentiation antigens Mart-1_{26-35}, $gp100_{209-217}$, $gp100_{280-288}$, $tyrosinase_{369-377}$ and the control antigen influenza virus58-66 in the blood lymphocytes and lesional skin lymphocytes (nevus with black arrow in Figure 1) of a patient with regressing nevi compared to patients with vitiligo.*

	Percentage of $CD3^+$ cells (%)		Percentage of $CD8^+$ cells (%)					
	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$ ratio	Mart-1_{26-35}	$Gp100_{209-217}$	$Gp100_{280-288}$	$Tyr_{369-377}$	Flu_{58-66}
Patient with regressing nevi Blood lymphocytes	41.6	42.6	0.98	0.18	0.11	1.09	0.07	0.02
Lesional lymphocytes	11.4	78.9	0.14	0.53	0.47	3.09	0.14	0.05
Vitiligo patients (positive controls)	/	/	/	0.36	0.18	1.00	0.11	0.07

lymphocytes from a biopsy of the regressing nevus using a 3-dimensional skin explant culture method according to Clark *et al*. (10). The T-cell population isolated from the regressing nevus showed a strongly reduced $CD4^+/CD8^+$ ratio, as compared to the peripheral blood (Table I).

As our patient was HLA-A2 positive, we were able to analyze the antigen specificity of the T cells using HLA-A2/peptide tetramers for the melanocyte differentiation antigens Mart-1_{26-35}, $gp100_{280-288}$, $gp100_{209-217}$, $tyrosinase_{369-377}$ and the control antigen influenza $virus_{58-66}$. In the $CD8^+$ lymphocytes from peripheral blood, an increased percentage of $gp100_{280-288}$ (1.09%) $CD8^+$ cells was found (Table I), as well as moderately increased levels of Mart1-specific T-cells. Among T-cells isolated from the regressing nevus, marked populations of $gp100_{280-288}$ (3.09%), $gp100_{209-217}$ (0.47%) and Mart-1_{26-35} (0.53%)-specific CD8 + T-cells were found, as compared to our previous analyses of skin-infiltrating T-cells in HLA-A2 + healthy donor skin (n=5), showing an average percentage of T-cells reactive with Mart-1 of 0.08%, 0.13% $gp100_{209-217}$, 0.38% $Gp100_{280-288}$-specific T-cells (11). The percentages of $tyrosinase_{369-377}$- and influenza $virus_{58-66}$-specific T-cells in the regressing nevus and in the blood were comparable to those of healthy donor skin (0.18% tyrosinase- and 0.03% flu-specific T-cells) (11). The percentages of antigen-specific T-cells were higher in the regressing nevus as compared to the peripheral blood lymphocytes of the same patient, indicating an enrichment of melanocyte antigen-reactive T-cells in the regressing nevus.

In parallel, we analyzed two patients with vitiligo as positive control patients with melanocyte-reactive autoimmunity. Both patients had an extensive form of generalized vitiligo [>60% body surface area (BSA)] and attended our Department with a request for complete skin depigmentation. The levels of antigen-specific T-cells found in the patient with regressing nevi were comparable to the melanocyte-reactive T-cell responses detected in the blood of the two vitiligo patients (Table I).

Discussion

Insights into the mechanisms leading to regression of melanocytic lesions is important for both pigmentation disorders and melanoma. The involvement of melanocyte-specific T-cells in the regression of nevi has been suggested by several authors (12). However, this is the first study that confirms the role of Mart-1 and gp100-specific $CD8^+$ cells in the pathogenesis of spontaneous regressing nevi by tetramer analysis. Besides a marked population of gp100- and Mart-1-positive cytotoxic T-cells in the regressing nevus, we also detected these T-cell clones in the peripheral blood of our patient. The patient had several histopathologic reports of immune reactions around dysplastic nevus cells. This finding supports the theory that a MDA-specific immune reaction may develop as a result of immunosurveillance and may offer protection against the development of neoplasia. This makes our patient exceptional since halo or regressing nevi are most frequently not associated with dysplastic nevi or melanoma.

Our results point to the involvement of a systemic immune reaction in the pathogenesis of multiple regressing nevi. Previously, an enhanced number of activated ($CD98^{bright}$) and cell proliferating ($CD71^{bright}$) lymphocytes was found in the blood of patients with halo nevi. These activated cells disappeared after excision of the halo nevus which supports the presence of a systemic reaction during the halo phenomenon (13). However, oligoclonal T-cell clones and melanocyte-specific T-cells have previously not been detected in the circulation in patients with spontaneous regressing nevi (6).

Despite a course of isotretinoin in 2003, the immune response observed in our patient is most likely to be spontaneous since no regression of nevi has been described due to isotretinoin treatment. Topical retinoids have been shown to induce inflammatory responses in dysplastic nevi sometimes leading to the complete regression of nevi but this has not been described for oral isotretinoin (14).

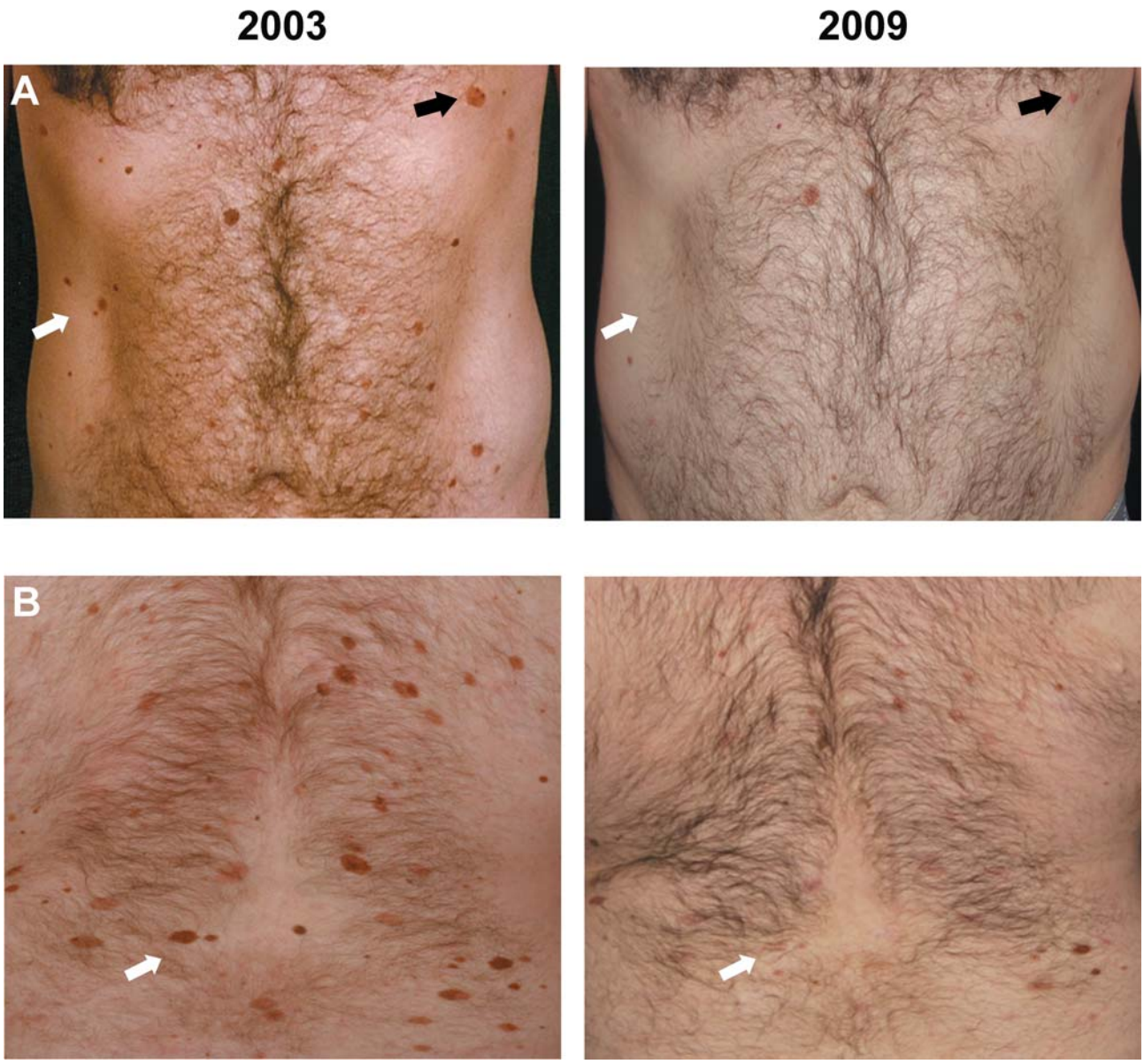

Figure 1. *Clinical images taken in 2003 and 2009 showing clear regression of almost all nevi on the trunk (A) and the back (B). The arrows show nevi that regressed almost completely.*

Nonetheless, some antitumoral and immunomodulating effects have been reported in combination treatments for melanoma containing isotretinoin (15, 16).

Mart-1 and gp100 vaccines are being developed to induce an effective antitumoral immune response in melanoma (17). Self-tolerance for these normal-expressed antigens may dampen MDA-specific immune responses. Nonetheless, in a subset of melanoma patients, spontaneous or vaccination induced MDA-specific T-cells lead in a subset of melanoma patients to vitiligo-like depigmentations which are associated with a better prognosis (18). A study of Jacobs *et al*. (19) in gp100-vaccinated melanoma patients found gp100- and Mart-1-specific T-cells both in regressing melanomas and vitiligo-like lesions, indicating that the induction of a MDA-specific immune response can indeed induce depigmentation. The role of preventive MDA-specific vaccination in patients at high risk for melanoma, such as patients with the atypical nevus syndrome, has not been investigated. As MDAs are also expressed on benign nevi, MDA-specific vaccination may also lead to immune-based elimination of melanocytic lesions.

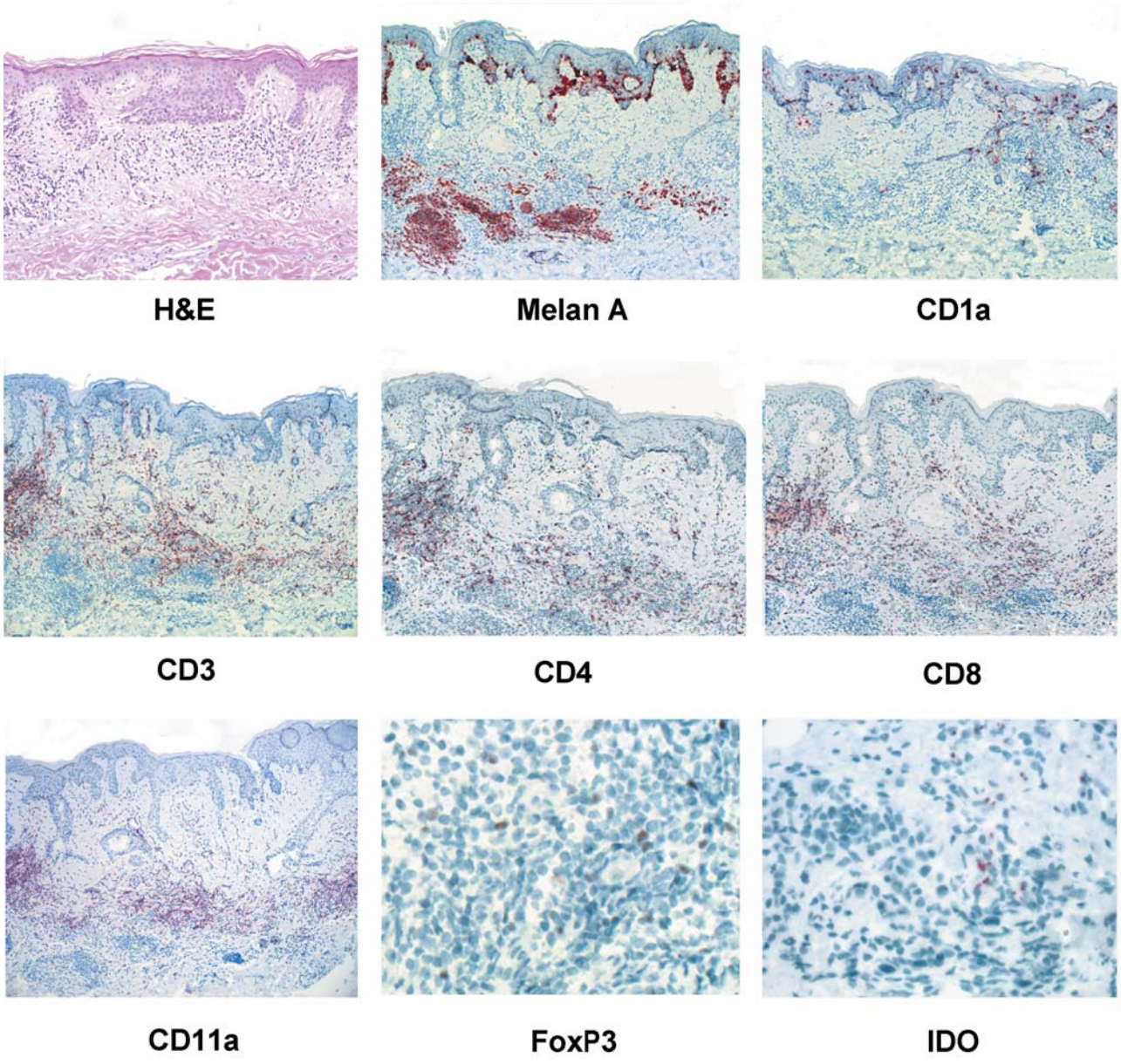

Figure 2. *Immunohistochemistry of a regressing nevus showing a marked CD3+, CD4+, CD8+, CD11a+ lymphocytic infiltrate and CD1a+ Langerhans cells around nevus cell nests. Limited FoxP3+ cells were present and expression of indoleamine 2,3-dioxygenase was observed. (nevus with black arrow in Figure 1).*

After Mart-1-vaccination, vitiligo-like depigmentations have been observed but these responses did not show a predominance to target nevoid melanocytes. Cassarino *et al*. (20) was unable to demonstrate an increased immune reaction in benign nevi after gp100 and tyrosinase peptide vaccinations in melanoma patients. Nonetheless, the MDA immune response observed in our patient with regressing nevi highlights the role of a melanocyte-specific immune response in benign regressing nevi, which supports the possible efficacy of MDA vaccination as a preventive strategy in patients with multiple dysplastic nevi.

The reason why some regressing nevi develop a halo and others do not, remains a topic of debate. Two main hypotheses have been proposed. The destruction of adjacent melanocytes could be due to the release of inflammatory cytokines during the regression of the nevus which may spread in a circular way resulting in a depigmented ring. A second hypothesis is that the immune response of T

lymphocytes is directed against antigens expressed both on nevus melanocytes and on surrounding epidermal melanocytes (21). As the regressing nevus in our study involved a nevus without halo which showed an infiltrate with Mart-1- and gp100-specific T-cells, this finding argues against the latter hypothesis, as these melanocyte differentiation antigens are also present in epidermal melanocytes, being normal proteins involved in the pigmentation process (22-24). Moreover, we observed the same T-cell clones in our vitiligo patients used as positive controls. These results suggest that MDA-specific lymphocytes are not only involved in the destruction of epidermal melanocytes as seen in vitiligo, but can also target specifically nevoid melanocytes (25, 26). As our patient showed no signs of depigmentation, the reason for immune-based destruction of benign melanocytes is unlikely to reside only in the development of MDA-specific cytotoxic T-cells. Besides MDA-specific immune reactions, local factors (*e.g.* antigen expression of melanocytes, trauma) may be necessary to induce depigmentation (27).

In conclusion, we found specific T-cell responses against gp100 and Mart-1 in a regressing nevus, as well as in the blood of a patient with multiple regressing nevi. This indicates that an effective systemic immune reaction against MDA-antigens is not limited to vitiligo patients or melanoma patients with regression. Furthermore, the presence of MDA-specific T-cells suggests that this is an example of an efficient early antitumoral response.

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