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**PD-1 EXPRESSION IN
PRIMARY CUTANEOUS LYMPHOMA**

Fatoş Çetinözman

PD-1 expression in primary cutaneous lymphoma

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PD-1 EXPRESSION IN PRIMARY CUTANEOUS LYMPHOMA

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to Marcel

who supported me in all possible ways

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1

GENERAL INTRODUCTION

INTRODUCTION

In recent years the molecule programmed death-1 (PD-1) has attracted much attention for several reasons. First, it is a critical element in the negative regulation of cellular immune responses. Second, blockage of the interaction between PD-1 expressed on T cells and its ligand PD-L1 expressed on tumor cells has emerged as a promising approach in the treatment of several cancers. Third, PD-1 is constitutively expressed by a subset of T cells, namely follicular helper T cells which play a key role in the differentiation of germinal center B cells into memory B cells or plasma cells. Fourth, expression of PD-1 has been used as important biomarker in the differentiation between different types of T-cell non-Hodgkin lymphoma. The main focus of the studies in this thesis was to determine whether expression of PD-1 might also be a useful marker in the diagnosis of different types of cutaneous lymphoma.

In this introductory chapter we will first describe general knowledge about the PD-1 molecule and the physiological role of PD-1 in cellular and humoral immunity. Next, expression of PD-1 in different types of nodal non-Hodgkin lymphoma and its diagnostic significance will be presented. Finally, primary cutaneous T-cell lymphoma and outstanding issues will be introduced and PD-1 related important contributions will be explained.

DISCOVERY AND DESCRIPTION OF THE PD-1 MOLECULE

PD-1 (CD279) was originally identified in 1992 by Ishida *et al.* who were studying dependency of programmed cell death on *de novo* RNA and protein synthesis. In mouse cell lines that were induced to undergo apoptosis they discovered that the PD-1 gene was activated and involved in this type of cell death.¹ Human full-length PD-1 was identified two years later as a type I trans-membrane glycoprotein of 288 amino acids (aa) and the PD-1 gene (named PDCD1) was mapped to chromosome 2q37.3.^{Ref 2} The extracellular regions of human and mouse PD-1 are relatively highly conserved (approximately 65% homology).³ PD-1 is considered to belong to the immunoglobulin (Ig) superfamily, because the structure of the extracellular domain resembles an Ig variable region (IgV). PD-1 is a monomer molecule and structurally related to family members CD28 and CTLA-4, which are functional as homodimer molecules.⁴ The extracellular domain of PD-1 is connected to a transmembrane region and an intracellular tail (Figure 1), containing two phosphorylation sites called ITSM (immunoreceptor tyrosine-based switch motif) and ITIM (immunoreceptor tyrosine-based inhibitory motif). Splice variants of the human PD-1 gene have been reported.⁵ One splice variant that lacks the transmembrane region represents the soluble form of PD-1, which can functionally antagonize the interaction of cell membrane PD-1 with its ligand.⁶

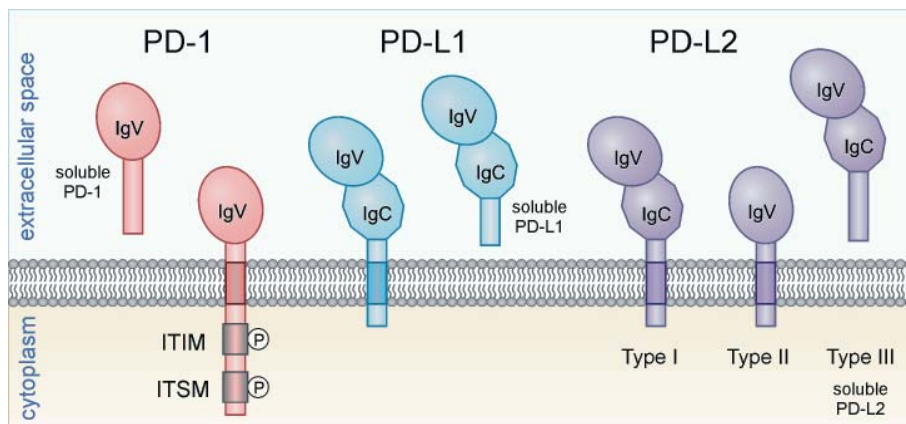


Figure 1. Structures of the PD-1 molecule and its ligands PD-L1 and PD-L2. PD-1 contains an extracellular Ig variable region (IgV)-like domain and an intracellular tail with two tyrosine-containing sites, called ITSM and ITIM, which are phosphorylated when PD-1 binds its ligand PD-L1 or PD-L2. The extracellular part of ligands PD-L1 and PD-L2 contains one IgV-like domain and one Ig constant region (IgC)-like domain and both have a relatively short intracellular domain without clear signaling motifs. In addition, PD-L2 (type II) contains only one IgV-like domain. The molecular structure of the soluble forms of PD-1 and ligands PD-L1 and PD-L2 are indicated as well.

DISCOVERY AND DESCRIPTION OF THE PD-1 LIGANDS

PD-1 has two ligands named PD-L1 (CD274; B7-H1) and PD-L2 (CD273; B7-DC). Human PD-L1, which was discovered in 2000, is 290 aa in size and is encoded by the *CD274* gene on chromosome 9.^{Ref 7} One year later PD-L2 was identified as a glycoprotein consisting of 273 aa and being encoded by the *PDCD1LG2* gene (Table 1).⁸ The *CD274* and *PDCD1LG2* genes are located next to each other and separated by only 42 kb of intervening genomic DNA. PD-L1 and PD-L2 share 40% aa identity. Both PD-1 ligands are type I transmembrane molecules and member of the Ig superfamily. Like PD-1, the extracellular part of PD-L1 and PD-L2 contains one IgV-like domain, but unlike PD-1, the extracellular part of PD-L1 and PD-L2 also contains one additional Ig constant region (IgC)-like domain (Figure 1). The intracellular domains of PD-L1 and PD-L2 are relatively short, only about 30 aa, without clear signaling motifs, but it is suggested that these intracellular tails may have a functional roles.⁹ PD-L2 has a two- to six-fold higher affinity for PD-1 than does PD-L1.^{Ref 10}

Ligands PD-L1 and PD-L2 also have splice variants and soluble forms with PD-1 binding capacity (Figure 1; Table 1),¹¹⁻¹³ but their role is unclear. Soluble PD-L1 is released by activated dendritic cells, but not by macrophages, monocytes or T cells.¹⁴ In addition, soluble PD-L1 is also produced by various tumor cells.¹⁴

Table 1. General facts on human PD-1 and ligands PD-L1 and PD-L2

	Discovery	Aliases	Gene name (chromosome location)	Size (amino acids)	Splice variants	Ligands
PD-1	1994	CD279	PDCD1 (2q37.3)	288	4	PD-L1 PD-L2
PD-L1	2000	CD274 B7-H1	CD274 (9p24.1)	290	1	PD-1 CD80
PD-L2	2001	CD273 B7-DC	PDCD1LG2 (9p24.2)	273	2	PD-1 RGMb

Although it is typically thought that PD-L1 and PD-L2 interact with PD-1, it is important to note that there are other binding partners for these two ligands (Table 1). PD-L1 has been shown to interact specifically with CD80 (B7-1).¹⁵ Keeping in mind that CD80 can also bind to CD28 and CTLA-4, it is difficult to comprehend how this complex receptor–ligand interactions is exactly balanced. Very recently, it is reported that PD-L2 can also bind to repulsive guidance molecule b (RGMb, also known as DRAGON),¹⁶ which was originally identified in the nervous system as a co-receptor for bone morphogenetic proteins and is expressed by macrophages and other cells of the immune system.¹⁷

EXPRESSION OF PD-1 AND ITS LIGANDS PD-L1 AND PD-L2

In humans, PD-1 can be expressed on T cells, B cells, natural killer T cells, monocytes and myeloid CD11c⁺ dendritic cells (DC) after activation.^{9;18} PD-1 is not present on naïve T cells, but is induced on CD4⁺ and CD8⁺ T cells after T-cell receptor-mediated activation and remains high in case of persistent stimulation with antigen (Table 2). In addition, PD-1 can also be induced on T cells by the so-called common gamma-chain cytokines interleukin 2 (IL-2), IL-7, IL-15 and IL-21, which are important for survival and expansion of T cells.¹⁹ PD-1 is highly expressed on non-functional so-called “exhausted” T cells,²⁰ which are the result of persistent antigen exposure, caused by chronic viral infections or progressive tumors.²¹ Remarkably, follicular helper T (TFH) cells, which are key regulators of germinal center B cell differentiation into plasma cells and memory B cells, have a constitutive high expression of PD-1.^{Ref} ²² Regulatory T (Treg) cells, which suppress immune system activation and promote immunologic tolerance, also have a constitutive high PD-1 expression.²³ Triggering of the B-cell receptor stimulate PD-1 expression on B cells.^{9;18} Freshly isolated NK cells from healthy donors do not express PD-1, but IL-2 can induce PD-1 expression on NK cells.²⁴ Freshly isolated immature Langerhans cells from healthy donors express PD-1, but lose this expression after maturation.²⁵

Table 2. Expression of PD-1, PD-L1 and PD-L2 on human cells *

	PD-1		PD-L1		PD-L2	
	Basal #	Activated #	Basal #	Activated #	Basal #	Activated #
CD4 ⁺ T cells	–	+++	–	+	–	+
CD8 ⁺ T cells	–	+++	–	+	–	+
Exhausted T cells	–	+++	nd	nd	nd	nd
CD4 ⁺ regulatory T cells	++	+++	+	+	–	–
CD4 ⁺ follicular helper T cells	++	+++	nd	+	–	–
B cells	–	++	nd	++	–	–
Monocytes	–	+	+	++	–	+
DC	–	+	+	+	–	++
Langerhans cells	+	–	+	++	+	++
Keratinocytes	–	–	–	+	–	–

* adapted from Keir *et al.* ⁹ and explained in detail in the text; nd: not determined

activation status of the cells: at baseline or after activation

PD-L1 expression is found on a broad range of immune cells (Table 2), including T cells and antigen-presenting cells, and is upregulated after activation.⁹ In particular type I and type II interferons can upregulate PD-L1 expression. PD-L1 can also be induced on a variety of non-immune cells, such as epithelial cells including keratinocytes and endothelial cells. Of note, tumor cells have been reported to express PD-L1 as well. Expression of PD-L2 has initially been reported to be restricted to activated professional antigen-presenting cells (Table 2), but recently it was shown that PD-L2 is expressed on activated human CD4⁺ and CD8⁺ T cells as well.²⁶

SUPPRESSION OF T-CELL FUNCTION BY THE PD-1/PD-L AXIS

T cells become activated when their T-cell receptor (TCR) recognizes an antigenic peptide–MHC complex that is presented by antigen-presenting cells. TCR are associated with CD3, which actually mediate the activation signal via activation of a series of kinases, such as ZAP70, PI3K, PKC- θ and AKT (Figure 2). T cells concomitantly receive multiple additional antigen-independent signals via receptor/ligand interactions, collectively called costimulation, which can deliver positive or negative signals.²⁷ Positive costimulation is essential for naïve T cells to become fully activated and if there is no or not enough positive signaling, naïve T cells fail to develop into effector or memory T cells and become nonresponsive to subsequent antigen stimulation, a situation which is often indicated with the term

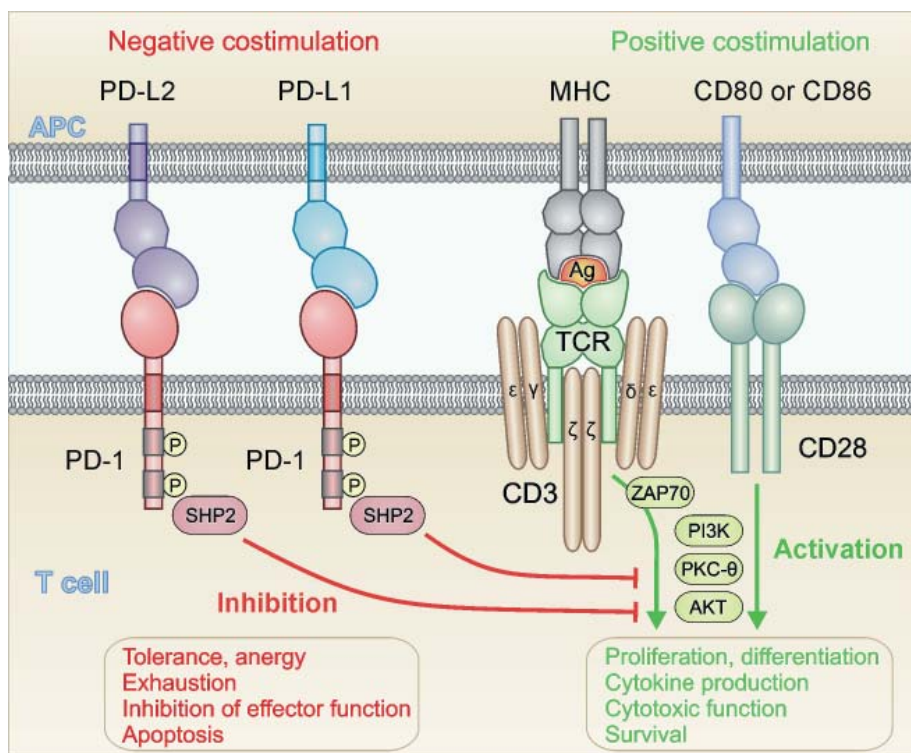


Figure 2. Inhibition of T-cell activation by PD-1. Antigen recognition by the T-cell receptor (TCR) provides an activation signal via CD3. Concomitant binding of CD28 to its ligand CD80 or CD86 strongly promotes T-cell activation, while ligation of PD-L1 or PD-L2 to PD-1 causes recruitment of phosphatase SHP-2 that disrupts the TCR and CD28 signaling pathway. A more detailed description is provided in the text.

anergy.²⁸ Predominance of negative co-stimulation will induce the development of regulatory T cells, T-cell tolerance or apoptosis. Receptors of the CD28/CTLA-4 family, including PD-1, have a key role in the outcome of T-cell responses, as these receptors provide potent costimulatory or inhibitory signals upon interaction with their corresponding ligands.

Costimulation is highly complex, because some of these receptor and ligands are constitutively expressed while others appear on the cell surface after activation, and in addition, some of the receptors have dual specificity or have similar specificity with different affinity (Figure 2). To provide a detailed description of the costimulatory and inhibitory signals is beyond the scope of this thesis, though an extensive overview can be obtained from references.²⁷ Only the PD-1/PD-L signaling will be described here, as PD-1 is the primary molecule of interest in this thesis.

Upon binding to its ligand, PD-1 becomes associated with TCR and CD28 microclusters that are assembled in the so-called immunological synapse.^{29,30} In addition, phosphorylation of the two intracellular tyrosine-based domains ITSM and ITIM takes place, which leads to the recruitment of phosphatases SHP-2 and possibly SHP-1 as well.⁹ Mutagenesis studies have shown that recruitment of SHP-2 by ITSM plays the primary functional role of PD-1 inhibition.³¹ The binding of SHP-2 by ITSM results in dephosphorylation of several molecules of the TCR signaling pathway (Figure 2).^{32,33} Altogether, triggering of PD-1 on the T cell leads to inhibition of proliferation and cytokine production, such as IL-2 (which protects against apoptosis), IFN- γ and TNF- α , and promotes T-cell apoptosis through inhibition of survival factor Bcl-xL.⁹

PD-1⁺ T FOLLICULAR HELPER CELLS AND B-CELL IMMUNITY

The differentiation of naïve B cells into memory B cells or antibody-secreting plasma cells in the germinal centers within the B-cell follicles is critically dependent on so-called T follicular helper (TFH) cells.^{34,35} In both humans and mice, TFH cells typically are CD4⁺ T cells with a high expression of chemokine receptor CXCR5 (chemokine C-X-C motif receptor 5) and CXCL13 (chemokine C-X-C motif ligand 13) and further express ICOS (inducible T cell costimulator), BCL6 (transcription factor B-cell lymphoma 6) and the cytokine IL-21. Of note, TFH cells have a constitutively high expression of PD-1, which is assumed to be one of the key instructive signals to guide the differentiation of germinal center B cells into memory or plasma cells.³⁶ These markers together are valuable to define TFH cells, but no single marker is sufficient to detect TFH cells as each of them can be found in other T cell subsets. Although TFH are defined by follicular location, T cells fulfilling the phenotypical criteria of TFH can also be detected outside the lymphoid organs, for example in the blood circulation. PD-1 is an important negative regulator of B-cell responses in the periphery. Binding of PD-1 to its ligand causes phosphorylation of the ITSM and ITIM domains of the B-cell receptor, causing recruitment of phosphatase SHP-2, leading to dephosphorylation of key molecules immediately downstream of the B-cell receptor, thereby suppressing the activation of the B cell.⁹ Blockade of PD-1 on B cells enhances antigen-specific antibody responses.³⁷

TUMORS AND THE PD-1/PD-L PATHWAY

In mouse models it has been demonstrated that PD-L1 expressing tumor cells are resistant to killing by CD8⁺ T cells via the engagement of PD-1 on the T cells by PD-L1 on the tumor cells (Figure 3). This PD-1/PD-L interaction resulted in direct

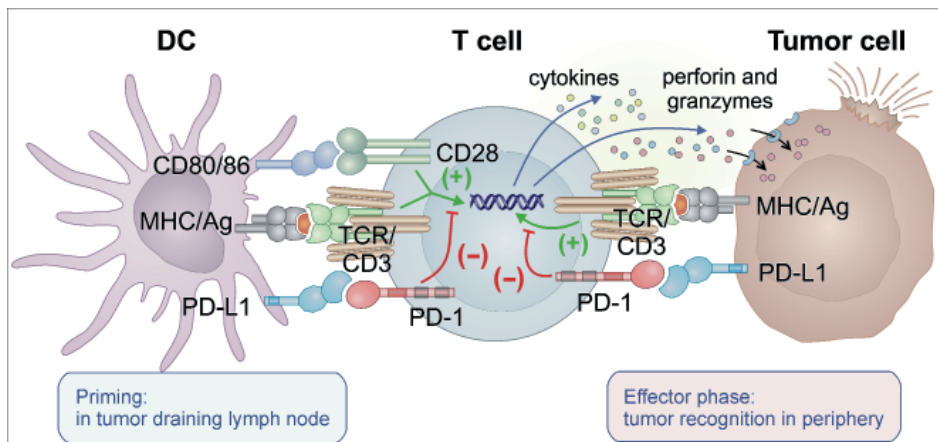


Figure 3. PD-1/PD-L pathway disrupts the anti-tumor T-cell response. Dendritic cells present tumor antigen to T cells to generate tumor-specific effector T cells. Both TCR-mediated signals and CD28 costimulation are necessary for optimal anti-tumor T-cell responses. Upon recognition of tumor antigen on tumor cells, the effector CD8⁺ T cell will secrete toxicity mediators perforin and granzymes to kill the tumor cell. Ligation of PD-1 causes the recruitment of phosphatase SHP-2 that disrupts the TCR and CD28 signaling pathway through direct dephosphorylation of signaling intermediates. Enhanced PD-1/PD-L expression results in inhibition of T-cell priming in the tumor draining lymph node. Tumors that express PD-L1 are able to defend themselves against the attacks of effector T cells, as they can trigger the PD-1 molecule on these T cells and thereby downregulate the effector functions of the T cells. Moreover, PD-L1 on tumor cells receive an anti-apoptotic signal from PD-1.

inhibition of the T-cell mediated cytotoxicity, while blockade of this interaction with antibodies restored the anti-tumor response.³⁸⁻⁴⁰ These data highlight that tumors can use the PD-1/PD-L pathway as evasion mechanism to escape from the immune system. Indeed, many different tumor types have upregulated PD-L1 expression⁴¹ and the majority of the tumor infiltrating lymphocytes show PD-1 expression.^{42,43} Therefore, the PD-1/PD-L pathway is generally considered to have a central role in the strategy of tumors to avoid eradication by the host defense system.

The exact mechanism why PD-L1 is expressed by tumor cells is currently unclear. Constitutive oncogene-driven expression of PD-L1 has been suggested, but alternatively, the PD-L1 expression might reflect the adaptation of the tumor to the anti-tumor immune response, a process termed adaptive resistance.⁴⁴ Several studies have investigated possible correlation of the expression of PD-1, PD-L1 and/or PD-L2 with patient prognosis, as reviewed by Sznol *et al.*⁴⁵ Because of the large variation and sometimes even opposite outcomes, these correlation studies should be interpreted with caution. Accumulating data from in vitro cell culture systems and animal models —indicating that PD-1/PD-L interactions form a major suppression mechanism within the tumor microenvironment— created the rationale to develop antibodies against PD-1 and PD-L1 for cancer immunotherapy.

Initial clinical studies using these antibodies demonstrated remarkable anti-tumor activity,^{45,46} validating the PD-1/PD-L pathway as target for cancer immunotherapy.

PD-1 EXPRESSION IN NODAL T-CELL NON-HODGKIN LYMPHOMA

Gene expression profiling analysis aimed to compare angioimmunoblastic T-cell lymphoma (AITL) with peripheral T-cell lymphoma, not otherwise specified (PTCL–NOS) clearly demonstrated that tumor cells in AITL show overexpression of several genes characteristic of normal TFH cells, such as *PDCD1* (encoding PD-1), *BCL6* and *CXCL13*. These findings strongly support that the TFH cell represent the cell of origin of AITL.⁴⁷ Multiple immunohistochemical studies on AITL specimens confirmed the (co)expression of one or more TFH markers.^{48–56} The pattern of PD-1 reactivity ranged from focal areas of increased numbers of extrafollicular T cells (usually less than 5% of interfollicular cells) to paracortical and diffuse reactivity throughout the lesion. PD-1⁺ cells were particularly enriched in areas unassociated with lymphoid follicles. The knowledge that AITL arise from these TFH cells provides a rational model to explain several of the peculiar pathological and biological features inherent to this disease, i.e. the expansion of B cells, the intimate association with germinal centers in early disease stages and the striking proliferation of follicular dendritic cells.

Apart from the cases which display peculiar pathological and biological features of AITL, some PTCL–NOS cases showed the expression of TFH markers and may be related to or derived from TFH cells. However, whether nodal follicular PTCL represents an early form of AITL or a distinct entity is unclear.^{57–59} PD-1 is not or rarely expressed on other types of T-cell non-Hodgkin lymphoma (NHL), such as anaplastic large cell lymphoma (ALCL) (Table 3).^{53–55;60}

PD-1 EXPRESSION IN NODAL B-CELL NON-HODGKIN LYMPHOMA

Several studies investigated PD-1 expression in nodal B-cell NHL, demonstrating that chronic lymphocytic leukemia (B-CLL) was the only subtype in which the malignant B cells exhibited PD-1 positivity in almost all cases.^{53;61} The strongest PD-1 staining was found in large para-immunoblasts and prolymphocytes located within proliferation centers, whereas small B cells in the surrounding areas showed less positivity. PD-1 is rarely expressed by the neoplastic B cells in follicular lymphoma and diffuse large B-cell lymphoma.^{51;53;61} Other subtypes of B-cell NHL exhibit no PD-1 positivity among the malignant cell population.

Table 3. PD-1 expression nodal T-cell non-Hodgkin lymphoma

Lymphoma type	Xerri et al. 2008	Yu et al. 2009	Krishnan et al. 2010	Matsumoto et al. 2010
Angioimmunoblastic T-cell lymphoma	8/8 (100%)	22/22 (100%)	74/80 (93%)	–
Peripheral T-cell lymphoma, not otherwise specified	0/5	0/10	10/14 (71%)	1/10 (10%)
Anaplastic large cell lymphoma	0/3	0/12	2/10 (20%)	–

B-cell NHL also comprise a variable proportion of PD-1⁺ reactive T cells,⁶¹⁻⁶⁵ showing significantly higher numbers of PD-1⁺ lymphocytes in follicular lymphoma than in diffuse large B-cell lymphoma.^{61,62} Several studies reported that increased numbers of PD-1⁺ T cells in nodal follicular B-cell lymphoma are associated with significantly improved survival, while in contrast, one other study showed that PD-1 positivity is associated with a poorer outcome.⁶¹⁻⁶⁴

PRIMARY CUTANEOUS LYMPHOMA

Definition

The term “primary cutaneous lymphoma” refers to a heterogeneous group of different T-cell and B-cell NHL that present in the skin without evidence of extra cutaneous involvement at the time of the diagnosis. It is important to differentiate these cutaneous T-cell lymphoma (CTCL) and cutaneous B-cell lymphoma (CBCL) from their nodal counterparts since they often have a completely different clinical behavior and prognosis and most often require different treatment modalities.

According to the World Health Organization – European Organization for Research and Treatment of Cancer (WHO-EORTC) classification,⁶⁶ primary cutaneous lymphoma are subdivided into the two main categories primary CTCL (75–80%) and primary CBCL (20–25%). Within the CTCL category the most common subgroups are: (1) the group of classical CTCL, including mycosis fungoides (MF), variants of mycosis fungoides and Sézary syndrome (SS); (2) the group of primary cutaneous CD30⁺ lymphoproliferative disorders (CD30⁺ LPD); (3) the group of rare and often aggressive cutaneous T/NK-cell lymphoma (Table 4). Within the CBCL category three main types of CBCL are described: primary cutaneous marginal zone lymphoma (PCMZL), primary cutaneous follicle center lymphoma (PCFCL) and primary cutaneous diffuse large B-cell lymphoma, leg type (PCLBCL-LT) (Table 4).

PD-1 expression in primary cutaneous T-cell lymphoma

Recent studies reported PD-1 expression in MF, SS and primary cutaneous small/medium sized pleomorphic T-cell lymphoma. In the following paragraphs these entities will be discussed and the potential significance of investigating PD-1 expression in these conditions will be presented.

• *Mycosis fungoides*

MF is the most common type of the CTCL, which mainly affects adults and accounts for almost 50% of all primary cutaneous lymphoma. MF is clinically characterized by the slow progression from patches to plaques, and even to tumors and in some cases may develop extracutaneous disease.⁶⁶

Histologically, the early stages of classic MF show superficial band-like or lichenoid infiltrates with atypical small- to medium-sized T cells with highly convoluted (cerebriform) and hyperchromatic nuclei. These atypical T cells might infiltrate into the epidermis (epidermotropism). With progression to tumor stage, the dermal infiltrates become more diffuse, with an increase in the proportion of tumor cells, as well as an increase in the number of blast cells, and the epidermotropism might disappear. The neoplastic T cells in MF commonly show a CD4⁺CD8⁻ phenotype, but may exhibit a CD4⁻CD8⁺ phenotype in some cases. Demonstration of an aberrant phenotype (for example, loss of pan-T-cell antigens, such as CD2, CD3, and CD5) is an important adjunct in the diagnosis of MF. Although MF commonly runs an indolent course, patients developing skin tumors, extracutaneous involvement or erythroderma may have a more unfavorable prognosis.

Wada *et al.* reported a percentage of more than 50% PD-1⁺ tumor cells in 6 of 15 cases of patch/plaque stage MF (40%), 9 of 15 cases of tumor stage MF (60%), and in 8 of 11 biopsy specimens from patients with SS (63%).⁶⁷ In the study of Kantekure *et al.* 16 of 26 MF biopsy specimens (62%) contained more than 25% PD-1⁺ tumor cells.⁶⁸ In this latter study, the authors showed that PD-1 was more frequently expressed at the early patch and plaque stages of CTCL, while PD-1 was expressed to a lesser extent at the tumor stage MF. Of note, this loss of PD-1 expression preferentially affected blast cells. Roncador *et al.* observed PD-1 expression in 5 of 9 MF cases (56%).⁵¹

• *Sézary syndrome*

SS is a leukemic form of CTCL defined by erythroderma, lymphadenopathy and clonal CD4⁺ T cells in skin and blood (Sézary cells), and generally having a poor prognosis with 25% 5-year disease specific survival. In addition, alopecia,

Table 4. WHO-EORTC classification of cutaneous lymphoma with primary cutaneous manifestations*

Cutaneous T-cell and NK-cell lymphoma
Mycosis fungoides
MF variants and subtypes
- Folliculotropic MF - Pagetoid reticulosis - Granulomatous slack skin
Sézary syndrome
Adult T-cell leukemia/lymphoma
Primary cutaneous CD30 ⁺ lymphoproliferative disorders
- Primary cutaneous anaplastic large cell lymphoma - Lymphomatoid papulosis
Subcutaneous panniculitis-like T-cell lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Primary cutaneous peripheral T-cell lymphoma, unspecified
- Primary cutaneous aggressive epidermotropic CD8⁺ T-cell lymphoma (provisional) - Cutaneous γ/δ T-cell lymphoma (provisional) - Primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma (provisional)
Cutaneous B-cell lymphoma
Primary cutaneous marginal zone lymphoma
Primary cutaneous follicle center lymphoma
Primary cutaneous diffuse large B-cell lymphoma, leg type

* adapted from Willemze et al. ⁶⁶

palmoplantar hyperkeratosis and onychodystrophy might be seen. The diagnosis of SS can be challenging. Especially in the early stages of the disease, it can be difficult to differentiate SS from erythrodermic inflammatory dermatoses (EID). Since the clinical presentation is generally not discriminative and histology may show reactive changes in up to one third of the cases, the diagnosis relies heavily on demonstration of neoplastic cells in the peripheral blood.^{69;70} In the WHO-EORTC classification for cutaneous lymphoma published in 2005, and incorporated in the WHO classification of lymphoid neoplasms in 2008, the diagnosis of SS is based on clinical presentation (erythroderma and lymphadenopathy) and demonstration of a T-cell clone in the peripheral blood (preferably the same clone in skin), in combination with one or more of the following criteria: an absolute Sézary cell count > 1000 cells per mm³; loss of T-cell markers CD2, CD3, CD4 and /or CD5 and /or an expanding population of CD4⁺ T cells leading to a CD4/CD8 ratio of more than 10. ^{Refs 58;66}

Samimi *et al.* found increased PD-1 expression in the peripheral blood neoplastic T cells in patients with SS, but not in the circulating T cells from patients with MF.⁷¹ They observed a strong decrease in PD-1 expression along with improvement of disease. Moreover, an increase in interferon gamma production was found in cultures of PBMC from patients with SS if the PD-1/PD-L1 interaction was blocked by anti-PD-1 antibodies. They suggested that the high PD-1 expression may contribute to immunosuppression in SS, but mechanisms involved are as yet unknown.⁷¹

• **Primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma**

Primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma (PCSM-TCL) is a CTCL that is included as a provisional entity and rare subtype of primary cutaneous peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) in recent cutaneous lymphoma classifications (WHO-EORTC 2005; WHO 2008).^{58,66} These PCSM-TCL characteristically present with a solitary plaque or tumor that is generally localized on the face or the upper trunk, and rarely with multiple papules, plaques or tumors.^{66,72,73} In particular patients presenting with a solitary skin lesion have an excellent prognosis.⁷⁴ Histologically, these lymphoma show nodular to diffuse infiltrates with a predominance of CD3⁺CD4⁺CD8⁻CD30⁻ small/medium-sized pleomorphic T cells and a small proportion (<30%) of large CD4⁺ T cells. In most cases there is a considerable admixture with small reactive CD8⁺ T cells, CD20⁺ B cells, including some blast cells, histiocytes, and in some cases plasma cells and eosinophils. The clinical presentation, the architecture and cellular composition of these PCSM-TCL are strikingly similar to those described previously in so-called pseudo-T-cell lymphoma.^{75,76} The relationship between PCSM-TCL presenting with a solitary lesion and pseudo-T cell lymphoma and the most appropriate term for such lesions is a matter of debate.

These PCSM-TCL should be distinguished clinically and histologically from other types of CTCL, in particular PTCL-NOS and tumor-stage MF, as they have a completely different prognosis and require different types of treatment.⁷⁴ Recently, Rodriguez-Pinilla *et al.* reported that medium-sized to large atypical CD4⁺ T cells in PCSM-TCL showed expression of PD-1, BCL6, CXCL13 and partially CD10, which suggested that these cells have a TFH cell phenotype.⁷⁷

PD-1 expression in primary cutaneous B-cell lymphoma

In the classification of WHO-EORTC (Table 4) three main types of CBCL are described: primary cutaneous marginal zone lymphoma (PCMZL), primary cutaneous follicle center lymphoma (PCFCL) and primary cutaneous diffuse large

B-cell lymphoma, leg type (PCLBCL-LT). PCMZL and PCFCL are indolent types of CBCL having excellent prognosis. PCLBCL-LT is an aggressive type of tumor with a 5-year survival of only circa 50%. Studies about PD-1 expression in CBCL is scarce. Rodriguez-Pinilla *et al.* reported negative staining for PD-1 in some cases of PCMZL and PCFCL.⁷⁷ However, studies on the number and the distribution of reactive T-cells as performed in nodal B-NHL are lacking.

AIMS AND OUTLINE OF THE THESIS

Studies in this thesis were aimed to investigate whether the PD-1 molecule a useful marker in the diagnosis of different types of cutaneous lymphoma. In our first study presented in **Chapter 2** we investigated the expression of PD-1 and other TFH cell markers in a large group of patients with PCSM-TCL. The goal of this study was to determine whether PD-1 expression by atypical T cells could serve as a useful diagnostic marker to differentiate between PCSM-TCL and other types of CTCL. In this study we included skin biopsies from patients with MF, cutaneous ALCL and cutaneous PTCL-NOS.

The study described in chapter 2 revealed that PD-1 expression was detected on neoplastic cells in only 2 of 21 MF biopsy samples. This result is in contrast to published studies of other research groups, which reported that PD-1 expression is commonly seen in MF. **Chapter 3** describes results of staining for PD-1 and other TFH markers (BCL-6, CXCL-13, CD10) in a large cohort of patients with MF, including patients with erythrodermic MF and SS.

It is well-known that histologic differentiation between SS and erythrodermic inflammatory dermatosis (EID) may be extremely difficult.⁷⁸ In **Chapter 4** we investigated the expression of PD-1 in skin biopsies of SS patients and patients with various types of EID. The main goal was to investigate if the number and distribution of PD-1⁺ T cells is useful as an adjunct in the differentiation between SS and EID.

In the continuous search for SS-specific diagnostic biomarkers, we explored in the study described in **Chapter 5** the expression of two other potential diagnostic markers, using the same group of patients with SS or EID as presented in chapter 4. These two markers are thymocyte selection-associated high mobility group box protein (TOX) and C-MYC, which is a transcription factor that regulates the global chromatin structure. Increased TOX expression by malignant CD4⁺ T cells

has been found in MF and SS, but information on TOX expression in EID is lacking. In addition, C-MYC positivity has been demonstrated in a considerable number of infiltrating lymphoid cells in MF and SS, while there is no information on C-MYC expression in EID. We explored in this study if expression of TOX and C-MYC can also be used as diagnostic marker for the differentiation between SS and EID.

In **Chapter 6**, we investigated the expression of PD-1 in skin sections from primary CBCLs. The aim of this study was to find out if PD-1 is expressed by the neoplastic B cells of these lymphoma. In addition, the number and distribution of PD-1⁺ T cells was investigated and correlated with clinical behavior.

Chapter 7 summarizes and discusses the findings described in the preceding chapters.

REFERENCES

1. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992;11:3887-3895.
2. Shinohara T, Taniwaki M, Ishida Y, Kawaichi M, Honjo T. Structure and chromosomal localization of the human PD-1 gene (PDCD1). *Genomics* 1994;23:704-706.
3. Cheng X, Veverka V, Radhakrishnan A et al. Structure and interactions of the human programmed cell death 1 receptor. *J Biol Chem* 2013;288:11771-11785.
4. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* 2005;23:515-548.
5. Nielsen C, Ohm-Laursen L, Barington T, Husby S, Lillevang ST. Alternative splice variants of the human PD-1 gene. *Cell Immunol* 2005;235:109-116.
6. Wu H, Miao M, Zhang G, Hu Y, Ming Z, Zhang X. Soluble PD-1 is associated with aberrant regulation of T cells activation in aplastic anemia. *Immunol Invest* 2009;38:408-421.
7. Freeman GJ, Long AJ, Iwai Y et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027-1034.
8. Latchman Y, Wood CR, Chernova T et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261-268.
9. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;26:677-704.
10. Zhang X, Schwartz JC, Guo X et al. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* 2004;20:337-347.
11. He XH, Xu LH, Liu Y. Identification of a novel splice variant of human PD-L1 mRNA encoding an isoform-lacking IgV-like domain. *Acta Pharmacol Sin* 2005;26:462-468.
12. Frigola X, Inman BA, Lohse CM et al. Identification of a soluble form of B7-H1 that retains immunosuppressive activity and is associated with aggressive renal cell carcinoma. *Clin Cancer Res* 2011;17:1915-1923.
13. He XH, Liu Y, Xu LH, Zeng YY. Cloning and identification of two novel splice variants of human PD-L2. *Acta Biochim Biophys Sin (Shanghai)* 2004;36:284-289.
14. Frigola X, Inman BA, Krco CJ et al. Soluble B7-H1: differences in production between dendritic cells and T cells. *Immunol Lett* 2012;142:78-82.
15. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. *Immunity* 2007;27:111-122.
16. Xiao Y, Yu S, Zhu B et al. RGMB is a novel binding partner for PD-L2 and its engagement with PD-L2 promotes respiratory tolerance. *J Exp Med* 2014;211:943-959.
17. Xia Y, Cortez-Retamozo V, Niederkofler V et al. Dragon (repulsive guidance molecule b) inhibits IL-6 expression in macrophages. *J Immunol* 2011;186:1369-1376.
18. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 2010;236:219-242.

19. Kinter AL, Godbout EJ, McNally JP et al. The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *J Immunol* 2008;181:6738-6746.
20. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH. Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J Exp Med* 2006;203:2223-2227.
21. Wherry EJ. T cell exhaustion. *Nat Immunol* 2011;12:492-499.
22. Chtanova T, Tangye SG, Newton R et al. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J Immunol* 2004;173:68-78.
23. Francisco LM, Salinas VH, Brown KE et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 2009;206:3015-3029.
24. Benson DM, Jr., Bakan CE, Mishra A et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood* 2010;116:2286-2294.
25. Pena-Cruz V, McDonough SM, Diaz-Griffero F, Crum CP, Carrasco RD, Freeman GJ. PD-1 on immature and PD-1 ligands on migratory human Langerhans cells regulate antigen-presenting cell activity. *J Invest Dermatol* 2010;130:2222-2230.
26. Messal N, Serriari NE, Pastor S, Nunes JA, Olive D. PD-L2 is expressed on activated human T cells and regulates their function. *Mol Immunol* 2011;48:2214-2219.
27. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* 2013;13:227-242.
28. Schwartz RH. T cell anergy. *Annu Rev Immunol* 2003;21:305-334.
29. Yokosuka T, Saito T. Dynamic regulation of T-cell costimulation through TCR-CD28 microclusters. *Immunol Rev* 2009;229:27-40.
30. Yokosuka T, Takamatsu M, Kobayashi-Imanishi W, Hashimoto-Tane A, Azuma M, Saito T. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J Exp Med* 2012;209:1201-1217.
31. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. *Proc Natl Acad Sci U S A* 2001;98:13866-13871.
32. Sheppard KA, Fitz LJ, Lee JM et al. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS Lett* 2004;574:37-41.
33. Parry RV, Chemnitz JM, Frauwirth KA et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol Cell Biol* 2005;25:9543-9553.
34. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011;29:621-663.
35. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol* 2013;13:412-426.
36. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* 2010;11:535-542.

37. Nicholas KJ, Zern EK, Barnett L et al. B cell responses to HIV antigen are a potent correlate of viremia in HIV-1 infection and improve with PD-1 blockade. *PLoS One* 2013;8:e84185.
38. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 2002;99:12293-12297.
39. Dong H, Strome SE, Salomao DR et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793-800.
40. Blank C, Brown I, Peterson AC et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res* 2004;64:1140-1145.
41. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat Rev Immunol* 2008;8:467-477.
42. Sfanos KS, Bruno TC, Meeker AK, De Marzo AM, Isaacs WB, Drake CG. Human prostate-infiltrating CD8+ T lymphocytes are oligoclonal and PD-1+. *Prostate* 2009;69:1694-1703.
43. Ahmadzadeh M, Johnson LA, Heemskerk B et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 2009;114:1537-1544.
44. Topalian SL, Drake CG, Pardoll DM. Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. *Curr Opin Immunol* 2012;24:207-212.
45. Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin Cancer Res* 2013;19:1021-1034.
46. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012;12:252-264.
47. de Leval L, Rickman DS, Thielen C et al. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood* 2007;109:4952-4963.
48. Dorfman DM, Brown JA, Shahsafaei A, Freeman GJ. Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol* 2006;30:802-810.
49. Grogg KL, Attygalle AD, Macon WR, Remstein ED, Kurtin PJ, Dogan A. Expression of CXCL13, a chemokine highly upregulated in germinal center T-helper cells, distinguishes angioimmunoblastic T-cell lymphoma from peripheral T-cell lymphoma, unspecified. *Mod Pathol* 2006;19:1101-1107.
50. Dupuis J, Boye K, Martin N et al. Expression of CXCL13 by neoplastic cells in angioimmunoblastic T-cell lymphoma (AITL): a new diagnostic marker providing evidence that AITL derives from follicular helper T cells. *Am J Surg Pathol* 2006;30:490-494.
51. Roncador G, Garcia Verdes-Montenegro JF, Tedoldi S et al. Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. *Haematologica* 2007;92:1059-1066.
52. Rodriguez-Pinilla SM, Atienza L, Murillo C et al. Peripheral T-cell lymphoma with follicular T-cell markers. *Am J Surg Pathol* 2008;32:1787-1799.

53. Xerri L, Chetaille B, Serriari N et al. Programmed death 1 is a marker of angioimmunoblastic T-cell lymphoma and B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia. *Hum Pathol* 2008;39:1050-1058.
54. Yu H, Shahsafaee A, Dorfman DM. Germinal-center T-helper-cell markers PD-1 and CXCL13 are both expressed by neoplastic cells in angioimmunoblastic T-cell lymphoma. *Am J Clin Pathol* 2009;131:33-41.
55. Krishnan C, Warnke RA, Arber DA, Natkunam Y. PD-1 expression in T-cell lymphomas and reactive lymphoid entities: potential overlap in staining patterns between lymphoma and viral lymphadenitis. *Am J Surg Pathol* 2010;34:178-189.
56. Zhan HQ, Li XQ, Zhu XZ, Lu HF, Zhou XY, Chen Y. Expression of follicular helper T cell markers in nodal peripheral T cell lymphomas: a tissue microarray analysis of 162 cases. *J Clin Pathol* 2011;64:319-324.
57. Van den Oord JJ, de Wolf-Peeters C, O'Connor NT, De vR, Tricot G, Desmet VJ. Nodular T-cell lymphoma. Report of a case studied with morphologic, immunohistochemical, and DNA hybridization techniques. *Arch Pathol Lab Med* 1988;112:133-138.
58. Swerdlow SH, Campo E, Harris NL et al. WHO classification of tumors - Haematopoietic and lymphoid tissues, Geneva, Switzerland. WHO PRESS 2008.
59. Jaffe ES, Nicolae A, Pittaluga S. Peripheral T-cell and NK-cell lymphomas in the WHO classification: pearls and pitfalls. *Mod Pathol* 2013;26 Suppl 1:S71-S87.
60. Matsumoto Y, Horiike S, Ohshiro M et al. Expression of master regulators of helper T-cell differentiation in peripheral T-cell lymphoma, not otherwise specified, by immunohistochemical analysis. *Am J Clin Pathol* 2010;133:281-290.
61. Muenst S, Hoeller S, Willi N, Dirnhofera S, Tzankov A. Diagnostic and prognostic utility of PD-1 in B cell lymphomas. *Dis Markers* 2010;29:47-53.
62. Carreras J, Lopez-Guillermo A, Roncador G et al. High numbers of tumor-infiltrating programmed cell death 1-positive regulatory lymphocytes are associated with improved overall survival in follicular lymphoma. *J Clin Oncol* 2009;27:1470-1476.
63. Wahlin BE, Aggarwal M, Montes-Moreno S et al. A unifying microenvironment model in follicular lymphoma: outcome is predicted by programmed death-1--positive, regulatory, cytotoxic, and helper T cells and macrophages. *Clin Cancer Res* 2010;16:637-650.
64. Richendollar BG, Pohlman B, Elson P, Hsi ED. Follicular programmed death 1-positive lymphocytes in the tumor microenvironment are an independent prognostic factor in follicular lymphoma. *Hum Pathol* 2011;42:552-557.
65. Alvaro T, Lejeune M, Salvado MT et al. Immunohistochemical patterns of reactive microenvironment are associated with clinicobiologic behavior in follicular lymphoma patients. *J Clin Oncol* 2006;24:5350-5357.
66. Willemze R, Jaffe ES, Burg G et al. WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005;105:3768-3785.
67. Wada DA, Wilcox RA, Harrington SM, Kwon ED, Ansell SM, Comfere NI. Programmed death 1 is expressed in cutaneous infiltrates of mycosis fungoides and Sezary syndrome. *Am J Hematol* 2011;86:325-327.

68. Kantekure K, Yang Y, Raghunath P et al. Expression patterns of the immunosuppressive proteins PD-1/CD279 and PD-L1/CD274 at different stages of cutaneous T-cell lymphoma/mycosis fungoides. *Am J Dermatopathol* 2012;34:126-128.
69. Sentis HJ, Willemze R, Scheffer E. Histopathologic studies in Sezary syndrome and erythrodermic mycosis fungoides: a comparison with benign forms of erythroderma. *J Am Acad Dermatol* 1986;15:1217-1226.
70. Trotter MJ, Whittaker SJ, Orchard GE, Smith NP. Cutaneous histopathology of Sezary syndrome: a study of 41 cases with a proven circulating T-cell clone. *J Cutan Pathol* 1997;24:286-291.
71. Samimi S, Benoit B, Evans K et al. Increased programmed death-1 expression on CD4+ T cells in cutaneous T-cell lymphoma: implications for immune suppression. *Arch Dermatol* 2010;146:1382-1388.
72. Grogg KL, Jung S, Erickson LA, McClure RF, Dogan A. Primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma: a clonal T-cell lymphoproliferative disorder with indolent behavior. *Mod Pathol* 2008;21:708-715.
73. Beltraminelli H, Leinweber B, Kerl H, Cerroni L. Primary cutaneous CD4+ small-/medium-sized pleomorphic T-cell lymphoma: a cutaneous nodular proliferation of pleomorphic T lymphocytes of undetermined significance? A study of 136 cases. *Am J Dermatopathol* 2009;31:317-322.
74. Bekkenk MW, Vermeer MH, Jansen PM et al. Peripheral T-cell lymphomas unspecified presenting in the skin: analysis of prognostic factors in a group of 82 patients. *Blood* 2003;102:2213-2219.
75. Rijlaarsdam JU, Scheffer E, Meijer CJ, Willemze R. Cutaneous pseudo-T-cell lymphomas. A clinicopathologic study of 20 patients. *Cancer* 1992;69:717-724.
76. Rijlaarsdam JU, Willemze R. Cutaneous pseudolymphomas: classification and differential diagnosis. *Semin Dermatol* 1994;13:187-196.
77. Rodriguez Pinilla SM, Roncador G, Rodriguez-Peralto JL et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol* 2009;33:81-90.
78. Ram-Wolff C, Martin-Garcia N, Bensussan A, Bagot M, Ortonne N. Histopathologic diagnosis of lymphomatous versus inflammatory erythroderma: a morphologic and phenotypic study on 47 skin biopsies. *Am J Dermatopathol* 2010;32:755-763.

2

EXPRESSION OF PROGRAMMED DEATH-1 IN PRIMARY CUTANEOUS CD4-POSITIVE SMALL/ MEDIUM-SIZED PLEOMORPHIC T-CELL LYMPHOMA, CUTANEOUS PSEUDO-T-CELL LYMPHOMA AND OTHER TYPES OF CUTANEOUS T-CELL LYMPHOMA

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ABSTRACT

In this study we investigated whether programmed death-1 (PD-1) could serve as useful diagnostic marker to differentiate between primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphomas (PCSM-TCL) and cutaneous pseudo-T-cell lymphomas on the one hand and other types of cutaneous T-cell lymphomas (CTCLs) on the other. Formalin-fixed, paraffin-embedded skin biopsies from 26 patients with PCSM-TCL or pseudo-T-cell lymphoma, including 1 patient with a lymphomatoid drug eruption, and 52 skin biopsies from other types of CTCLs were stained for PD-1. In addition, PD-1 positive cases were stained with antibodies against BCL6, CXCL13 and CD10 to determine a possible relationship with follicular helper T (TFH) cells. In all 26 cases of PCSM-TCL or pseudo-T-cell lymphoma, the medium-sized to large-sized atypical T cells consistently expressed PD-1, BCL6 and CXCL13, but not CD10. PD-1 expression was found in only 2 of 21 cases of mycosis fungoides and in only 2 of 16 cases of cutaneous peripheral T-cell lymphoma, unspecified. All 4 patients with an aggressive epidermotropic cytotoxic CD8⁺ CTCL and all 11 cases with a primary cutaneous CD30⁺ lymphoproliferative disorder were negative for PD-1. In conclusion, PD-1 is typically expressed by the atypical cells in PCSM-TCL and pseudo-T-cell lymphoma, but is not expressed or is rarely expressed in other types of CTCLs. Therefore, it may serve as a suitable adjunct in differential diagnosis. Our results demonstrate that the atypical cells in PCSM-TCL and pseudo-T-cell lymphomas share a common TFH phenotype and support the view that most cases classified nowadays as PCSM-TCL are identical to cutaneous pseudo-T-cell lymphomas described previously.

INTRODUCTION

Primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma (PCSM-TCL) is a cutaneous T-cell lymphoma (CTCL) that has been included as a provisional entity and rare subtype of primary cutaneous peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), in recent cutaneous lymphoma classifications [World Health Organization–European Organization for Research and Treatment of Cancer (WHO-EORTC) 2005; WHO 2008].^{22, 24} These PCSM-TCLs characteristically present with a solitary plaque or tumor that is generally localized on the face or the upper trunk and rarely present with multiple papules, plaques, or tumors.^{3, 10, 24} In particular, patients presenting with a solitary skin lesion have an excellent prognosis.² Histologically, these lymphomas show nodular-to-diffuse infiltrates with a predominance of CD3⁺, CD4⁺, CD8⁻, CD30⁻ small-sized to medium-sized pleomorphic T cells and a small proportion (<30%) of large CD4⁺ pleomorphic T cells. In most cases there is a considerable admixture with small reactive CD8⁺ T cells and CD20⁺ B cells, including some blast cells, histiocytes, and in some cases plasma cells and eosinophils. The clinical presentation, the architecture, and cellular composition of these PCSM-TCLs are strikingly similar to those described previously in so-called pseudo-T-cell lymphomas.^{16, 17} Demonstration of a T-cell clone (common) and loss of pan-T-cell antigens (rare) are nowadays used as diagnostic criteria for PCSM-TCL,¹ but delineation between PCSM-TCL and pseudo-T-cell lymphomas is arbitrary and a matter of debate.

Recently, Rodriguez-Pinilla *et al.* reported that the large atypical CD4⁺ T cells in PCSM-TCL express programmed death-1 (PD-1), BCL6 and CXCL13.¹⁸ PD-1, which is located on chromosome 2q37, is a member of the CD28/CTLA-4 receptor family that regulates cellular immune responses.^{7, 8} PD-1 has 2 known ligands called PD-L1 and PD-L2. Engagement of PD-1 by its ligands inhibits T-cell activation and induces peripheral tolerance.¹² PD-1 is constitutively expressed by a particular germinal center T-cell subset, called follicular helper T (TFH) cells. These TFH cells, which also highly express CXCL13 and BCL6,⁶ play an important role in germinal center formation and plasma cell development. The expression of PD-1, BCL6, and CXCL13 by the large atypical CD4⁺ T cells in PCSM-TCL suggests that this type of CTCL originates from TFH cells.¹⁸ Previous studies have also demonstrated a high expression of PD-1,^{13, 19, 25, 26, 28} CXCL13,^{19, 26, 28} BCL6,^{19, 27, 28} and CD10^{27, 28} by neoplastic T cells in angioimmunoblastic T-cell lymphoma (AITL), and AITL is now commonly considered as a tumor of TFH cells.

PCSM-TCL should be distinguished histologically not only from pseudo-T-cell lymphomas but also from other types of CTCLs, in particular PTCL-NOS and tumor-stage mycosis fungoides (MF). In rare cases with scattered CD30⁺ blast cells, a diagnosis of primary cutaneous CD30⁺ lymphoproliferative disease may even be considered. Finally, cases with a large B-cell component and/or many plasma cells

may be misdiagnosed as cutaneous lymphoid hyperplasia or cutaneous marginal zone B-cell lymphoma. Differentiation between PCDM-TCL and these other types of CTCLs is extremely important, as they have a completely different prognosis and require different types of treatment.² Studies on PD-1 expression in PTCL-NOS are few and showed conflicting results, with percentages of PD-1-positive cases varying between 0% and 71%.^{13, 15, 25, 26, 28} A recent study reported expression of PD-1 in skin biopsies of 15 of 30 cases (50%) of MF and 8 of 11 cases (73%) of Sézary syndrome.²³ Another study also found increased PD-1 expression by the circulating neoplastic T cells in Sézary syndrome and suggested that PD-1 may contribute to immunosuppression.²⁰

The aim of this study was to determine whether PD-1 could serve as a useful diagnostic marker to differentiate between PCDM-TCL and other types of CTCLs. In addition, we also investigated all PD-1-positive cases for expression of BCL6, CXCL13, and CD10 to determine whether these cells have a TFH-cell phenotype.

MATERIALS AND METHODS

Patients

Paraffin-embedded skin biopsies from 26 patients with a PCDM-TCL or with a pseudo-T-cell lymphoma (further collectively termed PCDM-TCL; see Discussion section) were selected from the cutaneous lymphoma database of the Leiden University Medical Center. This group included 1 patient with a lymphomatoid drug eruption reported previously.¹⁶ In addition, 10 patients with patch/plaque-stage MF, 11 patients with tumor-stage MF, 16 patients with a primary ($n = 9$) or secondary ($n = 7$) cutaneous PTCL-NOS, 4 patients with an aggressive epidermotropic cytotoxic CD8⁺ CTCL, which is another rare subtype of PTCL, and 11 with a primary cutaneous CD30⁺ lymphoproliferative disorder, including 5 patients with lymphomatoid papulosis and 6 cases with a primary cutaneous anaplastic large cell lymphoma (C-ALCL), were studied. In all cases the diagnosis was based on the criteria of the WHO-EORTC classification for primary cutaneous lymphomas²⁴ and had been confirmed by an expert panel of dermato(patho)logists and hematopathologists of the Dutch Cutaneous Lymphoma group.

Immunohistochemistry

Sections of formalin-fixed, paraffin-embedded tissue were dried overnight (37°C) and subsequently dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation with 0.3 % hydrogen peroxide in methanol. After antigen retrieval by boiling for 10 min in 10 mmol/L citrate buffer (pH 6.0), tissue sections were incubated overnight with antibodies against PD-1 (1:200, goat polyclonal

antibody AF1086), CXCL13 (1:400, goat polyclonal antibody AF801), both purchased from R&D Systems (Abingdon, UK), or BCL6 (1:50, monoclonal M-7211) from Dako, Glostrup, Denmark. The PD-1 and CXCL13 sections were then incubated with a Goat HRP-Polmer Kit from Biocare Medical (Concord, CA). The BCL6 sections were incubated with a BrightVision Poly-HRP Kit from Immunologic (Duiven, The Netherlands). Immunoreactivity was detected using diaminobenzidine reagents, and counterstaining was performed with Mayer haematoxylin. Detection of CD3, CD4, CD8, CD10, CD20, CD68, CD79a, and MIB-1 was performed by routine immunostaining. Human tonsil and 3 cases of AITL were used as positive controls for PD-1, BCL6, and CXCL13 stainings. Negative controls were obtained by omitting the primary antibody.

T-cell Clonality Studies

Polymerase chain reaction was performed to analyze the clonal expansion of T cells. DNA was extracted from paraffin sections, and T-cell clonality was detected by analysis of T-cell receptor (TCR) β ($V\beta$ -J β and D β -J β) and TCR γ gene rearrangement, as previously described.⁴ Appropriate positive and negative controls were included in all experiments.

RESULTS

PCSM-TCL

Clinical features

The study group contained 18 male and 8 female patients with a median age of 55 years at presentation (range, 5 to 88 years). None of the 26 patients had skin lesions or a history suggestive of MF, lymphadenopathy, or B symptoms. One of these patients presented with 3 slightly scaling plaques on the trunk while using carbamazepine. As discontinuation of the antiepileptic drug resulted in complete clearance of the skin lesions, a diagnosis of lymphomatoid drug eruption was considered most likely.¹⁶ The etiology in the other 25 patients is unknown. Altogether, 23 of 26 patients presented with a solitary plaque or tumor (Figures 1A and 2A), which was preferentially localized on the head/neck ($n = 7$) or upper trunk ($n = 14$). Only 3 of 26 patients presented with multiple skin lesions. Routine physical and laboratory examinations showed no abnormalities in any case. Additional imaging studies had been conducted in only 4 patients and had not shown any abnormalities. In most cases treatment consisted of topical or intralesional steroids ($n = 10$) or excision ($n = 11$), whereas 1 patient was treated with radiotherapy. Spontaneous remission after the diagnostic skin biopsy or after discontinuation of the eliciting drug was observed in 4 patients. Local recurrences after treatment with

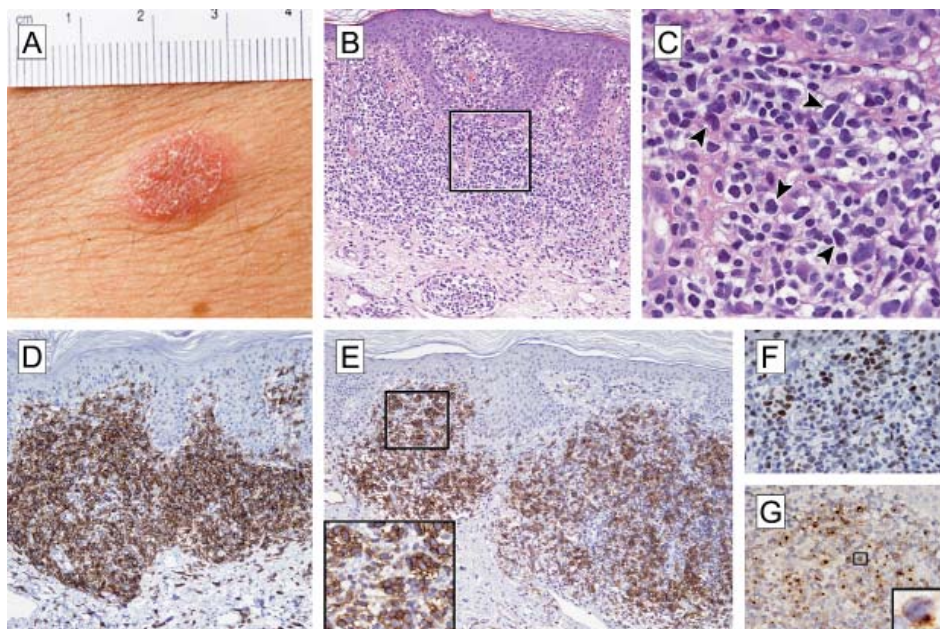


Figure 1. A PCSM-TCL patient with a superficial band-like pattern. (A) Patient presenting with a solitary plaque on the upper back. (B) Histologically, there is a subepidermal, band-like infiltrate. Panel C is a higher magnification of the indicated area depicted in panel B, showing medium-sized to large atypical cells (arrowheads). The atypical cells express CD4 (D), PD-1 (E), BCL6 (F), and CXCL13 as perinuclear dots (see inset) (G). The insert at the bottom of E is a higher magnification of the area indicated in the papillary dermis.

topical steroids were observed in 4 patients, 1 of them with multiple skin lesions. After a median follow-up of 10 months (range, 2 to 108 months), 23 patients are in complete remission, and 3 patients are alive with disease. None of the patients have developed extracutaneous disease.

Histology

Eleven cases, including the lymphomatoid drug eruption, showed a dense subepidermal band-like infiltrate, which characteristically showed a sharp demarcation at the lower border, which sometimes extended around hair follicles (Figures 1B and C). Fifteen cases showed a nodular-to-diffuse infiltrate throughout the entire dermis, often extending into the subcutaneous fat (Figures 2B and C). In both groups, infiltration of the epidermis or follicular structures was either not present or only focally present. The cellular composition of both groups was also highly similar. In all cases, the infiltrates were predominantly composed of small/medium-sized lymphocytes with variable numbers (5 to 25%) of medium-sized to

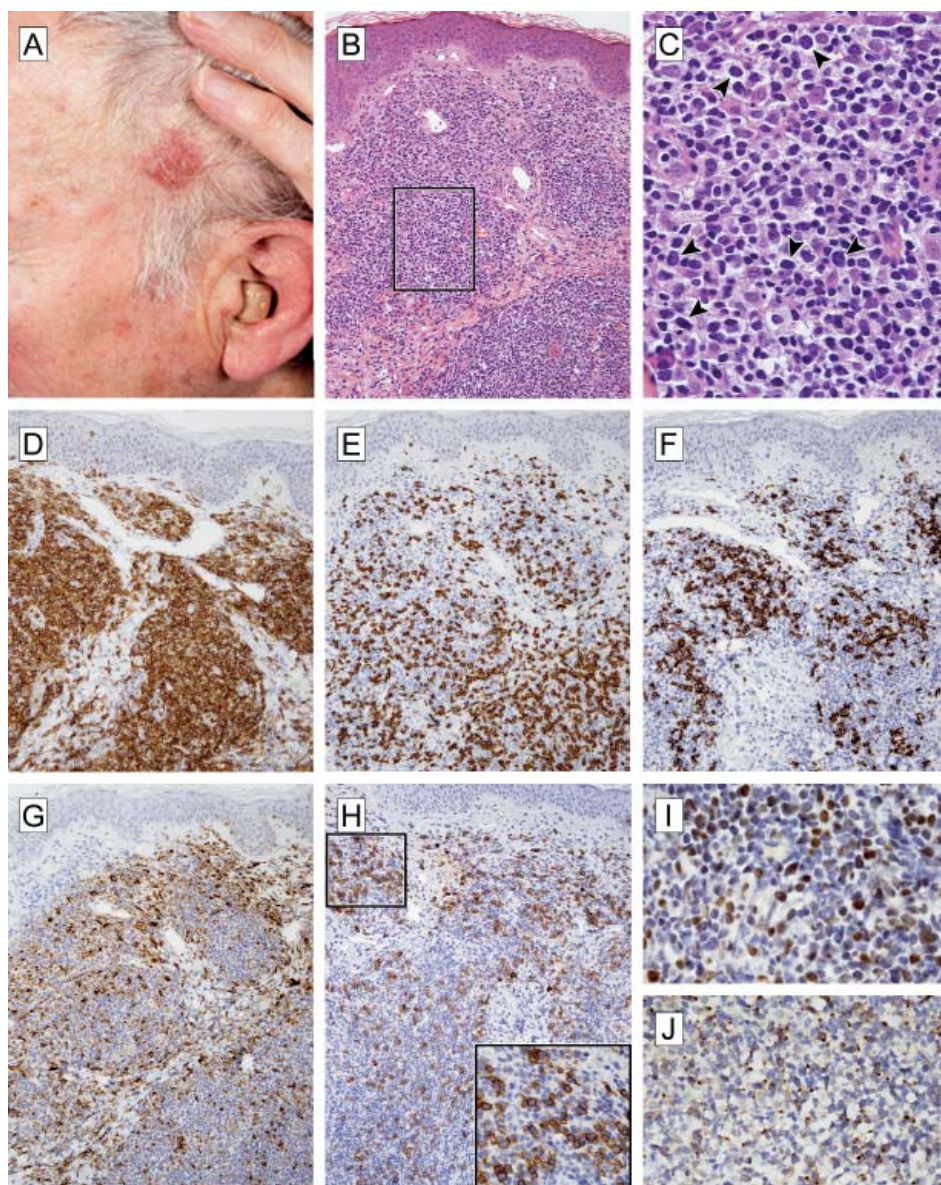


Figure 2. A PCSM-TCL patient with a nodular pattern. (A) Patient presenting with a tumor on the left temple (B) Histologically, the lesion shows a nonepidermotropic nodular infiltrate throughout the entire dermis. Panel C is a higher magnification of the marked area in panel B, showing many medium-sized to large atypical cells (arrowheads), which express CD4 (D). There is a considerable admixture with reactive CD8⁺ T cells (E), CD20⁺ B cells (F), and CD68⁺ histiocytes (G). The medium-sized to large atypical T cells express PD-1 (H), BCL6 (I) and CXCL13 (J). The insert at the bottom of H is a higher magnification of the area indicated in the papillary dermis.

large lymphoid cells with hyperchromatic and irregular nuclei and scattered blast cells (Figures 1C and 2C). Scattered mitotic figures were observed. In all cases there was a considerable admixture with histiocytes, and in some cases were accompanied by multinucleated giant cells and/or granulomatous changes. Plasma cells were generally present (in 3 cases even in large numbers), but eosinophils were few or absent.

Immunohistochemistry

In all cases, the medium-sized and large atypical cells, varying between 5% and 25% of the total infiltrate, showed a CD3⁺, CD4⁺, CD8⁻ T-cell phenotype without loss of CD2 or CD5 (Figures 1D and 2D). In all cases there was a considerable admixture with reactive CD8⁺ T cells (median, 20%; range, 5% to 30%), scattered or clustered CD20⁺ and/or CD79a⁺ B cells, including a few blast cells (median, 15%; range, 5% to 30%), and CD68⁺ histiocytes (median, 15%; range, 5% to 20%) (Figures 2E–G). Cytotoxic proteins were not expressed. CD30⁺ blast cells were generally few (< 5%). The proliferation rate varied between < 5% and 20% (median, 10%). MIB-1 was predominantly expressed by the large atypical cells. In all 26 cases the medium-sized to large atypical T cells consistently expressed PD-1, BCL6 and CXCL13 (Figures 1E–G and Figures 2H–J). In all 12 cases the CD10 staining was negative. Serial sections showed that CXCL13 generally stained 50% to 75% of PD-1⁺ cells, and BCL6 stained 25% to 50% of PD-1⁺ cells. In addition to the medium-sized to large atypical T cells, a few small reactive T cells showed a positive staining for PD-1, BCL6, and CXCL13. The neoplastic cells of the 3 AITL cases included as a positive control consistently expressed PD-1, BCL6, CXCL13, and CD10.

Molecular studies

Multiplex polymerase chain reaction analysis using the BIOMED-2 protocol demonstrated clonal TCR gene rearrangement in 16 cases, including the case with a lymphomatoid drug eruption, an oligoclonal pattern in 1 case, and an absence of clones in 5 cases. In 4 cases, evaluation was impossible because of poor DNA quality or lack of material.

PD-1 staining in other CTCLs

PCSM-TCL with a nodular or diffuse pattern resemble cutaneous PTCL–NOS and tumor-stage MF, whereas PCSM-TCL with a band-like pattern resemble patch/plaque-stage MF.^{1, 16, 17, 21} Skin sections from both plaque-stage and tumor-stage MF and from patients with cutaneous PTCL–NOS were therefore stained for PD-1 to determine whether this molecule is differentially expressed in these lymphomas. Because of the presence of scattered CD30⁺ cells in most PCSM-TCLs, which may raise suspicion to a cutaneous CD30⁺ lymphoproliferation, skin biopsies from 5

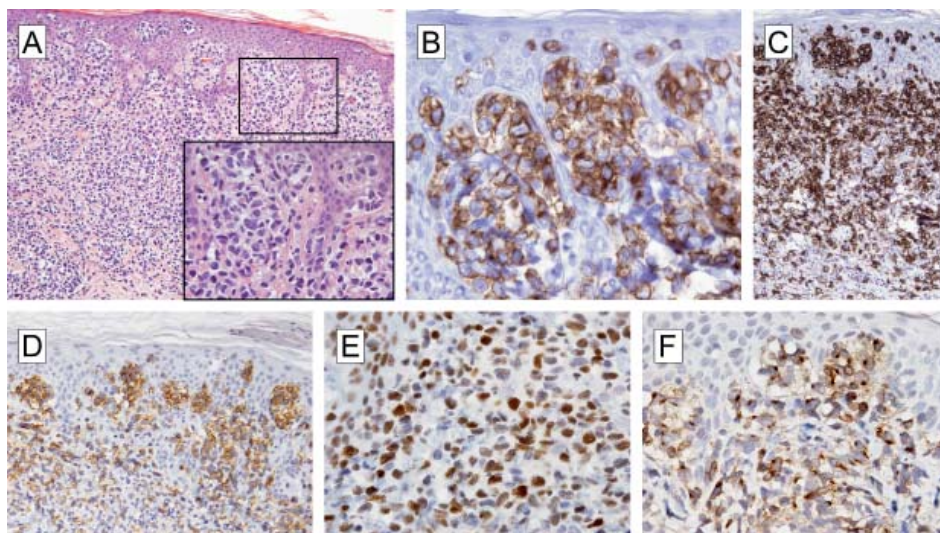


Figure 3. Mycosis fungoides. (A) Haematoxylin-eosin staining of a skin lesion from a patient with plaque-stage MF showing a dense infiltrate in the papillary dermis. The insert at the bottom is a higher magnification of the area indicated in the papillary dermis. There is extensive infiltration in the epidermis with formation of Pautrier microabscesses. The neoplastic cells expressed CD3 (B), CD10 (C), PD-1 (D), BCL6 (E), and CXCL13 (F).

patients with lymphomatoid papulosis (LyP) and 6 patients with a C-ALCL were selected as well.

The neoplastic cells in all 21 MF lesions had a CD3⁺, CD4⁺, CD8⁻ T-cell phenotype with frequent loss of pan-T-cell antigens in tumorous lesions (Figures 3A and B). In only 1 of 10 plaques and 1 of 11 tumorous lesions did virtually all neoplastic cells express PD-1 (Figure 3D). In both cases, BCL6 and CXCL13 were expressed by 25% to 50% of PD-1⁺ cells (Figures 3E and F), whereas CD10 was strongly expressed by the neoplastic T cells in 1 of these 2 cases (Figure 3C). In the other 19 MF biopsies, PD-1, BCL6, and CXCL13 only stained a few small reactive T cells.

Routine immunophenotypical studies in the PTCL-NOS group showed that 8 cases had a CD3⁺, CD4⁺, CD8⁻ T-cell phenotype, 4 cases a CD4⁻, CD8⁻ T-cell phenotype and 4 cases a CD4⁻, CD8⁺ T-cell phenotype. In contrast to the group of PCSM-TCLs, in most cases there was loss of 1 or more pan-T-cell antigens, a minimal admixture with inflammatory cells, and a proliferation rate of more than 50%. In only 2 cases, both secondary cutaneous CD4⁺ PTCL-NOS, did neoplastic T cells weakly stain for PD-1, BCL6, and CXCL13 but not for CD10. In the other cases, PD-1, BCL6, and CXCL13 only stained a few scattered reactive T cells. The neoplastic CD8⁺ T cells in 4 patients with an aggressive CD8⁺ epidermotropic cytotoxic CTCL did not express PD-1.

Consistent with previous studies, the large atypical CD30⁺ cells in skin lesions of 11 patients with LyP (n = 5) or C-ALCL (n = 6) were consistently negative for PD-1. PD-1 expression in the different types of CTCLs are summarized in Table 1.

DISCUSSION

In this study, the medium-sized to large atypical CD4⁺ T cells in all 26 cases of PCSM-TCL consistently showed expression of PD-1, BCL6, and CXCL13, which confirms the results of a previous study and suggests that these cells have a TFH cell phenotype. In contrast to AITL,²⁷ now commonly considered as a tumor of TFH cells, CD10 was not expressed in any of the 11 PCSM-TCLs studied. In the study by Rodriguez-Pinilla *et al.*, CD10 was expressed by a proportion of the atypical T cells in 3 of 15 cases.¹⁸ Interestingly, in our study PD-1 expression was found in only 2 of 16 PTCL-NOS, both cases with secondary cutaneous involvement, and not in the 4 aggressive epidermotropic cytotoxic CD8⁺ CTCLs. Our observations are consistent with other studies in PTCL-NOS, in which no^{25, 26} or a single case¹⁵ showed PD-1 positivity but are in contrast with another study in which 10 of 14 cases expressed PD-1.¹³ Moreover, in our study, PD-1 expression was found in only 2 of 21 cases of MF, including 1 of 10 plaque-stage and 1 of 11 tumor-stage MFs. Both cases also expressed BCL6 and CXCL13, whereas CD10 was strongly expressed in only 1 of them. A recent study reported expression of PD-1 in 6 of 15 (40%) skin biopsies of patch/plaque-stage MF and 9 of 15 (60%) biopsies of tumor-stage MF.²³ An explanation for these discrepant results is currently lacking. Consistent with previous studies, PD-1 was not expressed by the large CD30⁺ cells in LyP or C-ALCL.^{13, 25, 26}

The differences in PD-1 expression between PCSM-TCL on the one hand and cutaneous PTCL-NOS and tumor-stage MF on the other hand indicate that a positive staining of this marker may be used as an additional criterion to differentiate PCSM-TCL from these other types of CTCLs. This is particularly important in cases presenting with a solitary tumor, which was observed in all but 3 cases of PCSM-TCL and also in 4 patients with a primary cutaneous PTCL-NOS, all being negative for PD-1. In contrast to the patients with a PCSM-TCL, 3 of these 4 patients died of their disease 8 to 12 months after diagnosis, whereas 1 recently diagnosed patient is still alive with disease 5 months after diagnosis.

PCSM-TCL or cutaneous pseudo-T-cell lymphoma?

In this study, the group of PCSM-TCL contained cases that might be classified either as PCSM-TCL or as pseudo-T-cell lymphoma. The relationship between both conditions is a matter of debate. In the 1990s we and others introduced the term pseudo-T-cell lymphoma for lesions with histologic features suggesting a CTCL

Table 1. PD-1 Expression in Different Types of Cutaneous T-cell Lymphoma

Type of Lymphoma	Number	Cases With Neoplastic PD-1 ⁺ Cells	
		Neoplastic T cells	Percentage
PCSM-TCL / cutaneous pseudo-T-cell lymphoma	26	26	100
MF			
Patch/plaque stage	10	1	10
Tumor stage	11	1	9
PTCL-NOS			
Primary cutaneous	9	–	0
Secondary cutaneous	7	2	28
Aggressive CD8 ⁺ epidermotropic cytotoxic CTCL	4	–	0
Primary Cutaneous CD30 ⁺ lymphoproliferative disorders			
Lymphomatoid papulosis	5	–	0
Cutaneous anaplastic large cell lymphoma	6	–	0

but with a clinical presentation and clinical course more consistent with a benign condition.^{16, 17, 21} Prototypic patients were those using anti-epileptic drugs and presenting with a solitary plaque or nodule, which showed an atypical band-like subepidermal infiltrate mimicking MF. The correct diagnosis in such patients is a lymphomatoid drug eruption. For skin lesions showing the same atypical band-like infiltrate, but without known cause (most of them), the term pseudo-T-cell lymphoma was used.^{16, 17, 21} In addition to cases with a superficial band-like infiltrate, other patients presenting with a solitary plaque or nodule showed a nodular-to-diffuse infiltrate, which sometimes extended into the subcutaneous fat. Apart from the difference in architecture (band-like versus nodular to diffuse), these pseudo-T-cell lymphomas with a nodular-to-diffuse growth pattern showed the same clinical presentation and clinical course and had the same cellular composition as the cases with a band-like infiltrate: an atypical infiltrate with a predominance of small/medium-sized lymphocytes with variable numbers (but always < 30%) of medium-sized to large CD3⁺, CD4⁺, CD8[–] T cells, a considerable admixture with CD8⁺ T cells, CD20⁺ B cells, and histiocytes, a low proportion of proliferating cells, and no or only focal infiltration of the epidermis or follicular epithelium.¹⁶

In our initial studies, using Southern blot analysis, no clonality was found, and absence of clonality and absence of marker loss were suggested as useful criteria to differentiate these pseudo-T-cell lymphomas from MF and from primary cutaneous PTCL-NOS.¹

With the introduction of new and more sensitive techniques to demonstrate T-cell clonality it has become clear that most cases until then classified as pseudo-T-cell lymphoma contain clonal T cells. In addition, clonal T cells can also be found in lymphomatoid drug eruptions,^{5, 11, 14} as illustrated by the patient with a carbamazepine-associated lymphomatoid drug eruption in this study. In cases

in which a cause is known or presumed, and more in general in cases with an atypical band-like infiltrate confined to the papillary dermis, a diagnosis of pseudo-T-cell lymphoma or atypical lymphoid hyperplasia is often preferred, despite the detection of clonal T cells. However, in cases with a nodular-to-diffuse infiltrate, in which generally no causal factor can be detected, demonstration of T-cell clonality, in combination with a sometimes highly atypical morphology, nowadays generally results in a diagnosis of PCSM-TCL.

In this study, using the BIOMED-2 protocol, clonal TCR gene rearrangements were detected in 8 of 10 cases with a band-like pattern and 9 of 12 cases with a nodular-to-diffuse pattern. Both histologic groups had an identical cellular composition and in both groups the medium-sized to large atypical CD4⁺ T cells consistently expressed PD-1, BCL6, and CXCL13. In our view, there is therefore no reason to classify these two groups separately. Whether all cases should be classified as PCSM-TCL or as pseudo-T-cell lymphoma is a matter of debate. The overlapping features between PCSM-TCL and pseudo-T-cell lymphomas are widely recognized. Since differentiating criteria are lacking, Cerroni suggested the term “cutaneous nodular proliferation of pleomorphic T lymphocytes of undetermined significance”, and emphasized that these cases should not be treated aggressively.³ Most cases in our study were still classified as pseudo-T-cell lymphoma or as “spectrum of pseudo-T-cell lymphoma/PCSM-TCL”. Regardless of the term preferred, there is consensus that patients presenting with a solitary lesion and the characteristic histologic and immunophenotypical features described herein have an excellent prognosis. In our department, staging procedures are generally not performed in these patients, and their skin lesions, if not resolved spontaneously after skin biopsy, are treated with intralesional steroids or surgical excision and only by exception with radiotherapy. As in other pseudolymphomas, we advise a follow-up period of two years. The fact that all patients had an uneventful follow-up and that a skin relapse was observed in only 4 of 26 cases suggest that this is a correct and safe approach.

PCSM-TCLs that do not meet the criteria of the cases described herein are rare and should be fully staged. A recent study suggesting that PCSM-TCLs with rapidly growing bulky tumors, a low percentage of admixed CD8⁺ T cells, and/or a high proliferative fraction are at risk to develop progressive disease awaits further confirmation.⁹ Whether such cases have also a TFH phenotype remains to be established.

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REFERENCES

1. Bakels V, van Oostveen JW, van der Putte SC, et al. Immunophenotyping and gene rearrangement analysis provide additional criteria to differentiate between cutaneous T-cell lymphomas and pseudo-T-cell lymphomas. *Am J Pathol.* 1997;150:1941-9.
2. Bekkenk MW, Vermeer MH, Jansen PM, et al. Peripheral T-cell lymphomas unspecified presenting in the skin: analysis of prognostic factors in a group of 82 patients. *Blood.* 2003;102:2213-9.
3. Beltraminelli H, Leinweber B, Kerl H, et al. Primary cutaneous CD4+ small-/medium-sized pleomorphic T-cell lymphoma: a cutaneous nodular proliferation of pleomorphic T lymphocytes of undetermined significance? A study of 136 cases. *Am J Dermatopathol.* 2009;31:317-22.
4. Bruggemann M, White H, Gaulard P, et al. Powerful strategy for polymerase chain reaction-based clonality assessment in T-cell malignancies Report of the BIOMED-2 Concerted Action BHM4 CT98-3936. *Leukemia.* 2007;21:215-21.
5. Callot V, Roujeau JC, Bagot M, et al. Drug-induced pseudolymphoma and hypersensitivity syndrome. Two different clinical entities. *Arch Dermatol.* 1996;132:1315-21.
6. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* 2011;29:621-63.
7. Fife BT, Pauken KE. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Ann N Y Acad Sci.* 2011;1217:45-59.
8. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev.* 2010;236:219-42.
9. Garcia-Herrera A, Colomo L, Camos M, et al. Primary cutaneous small/medium CD4+ T-cell lymphomas: a heterogeneous group of tumors with different clinicopathologic features and outcome. *J Clin Oncol.* 2008;26:3364-71.
10. Grogg KL, Jung S, Erickson LA, et al. Primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma: a clonal T-cell lymphoproliferative disorder with indolent behavior. *Mod Pathol.* 2008;21:708-15.
11. Haddad A, Marek O, Noguerra ME, et al. Pseudo T-cell lymphoma with clonal expansion related to hypersensitivity to amoxicillin. *Haematologica.* 2005;90:ECR03.
12. Jin HT, Ahmed R, Okazaki T. Role of PD-1 in regulating T-cell immunity. *Curr Top Microbiol Immunol.* 2011;350:17-37.
13. Krishnan C, Warnke RA, Arber DA, et al. PD-1 expression in T-cell lymphomas and reactive lymphoid entities: potential overlap in staining patterns between lymphoma and viral lymphadenitis. *Am J Surg Pathol.* 2010;34:178-89.
14. Magro CM, Crowson AN, Kovatich AJ, et al. Drug-induced reversible lymphoid dyscrasia: a clonal lymphomatoid dermatitis of memory and activated T cells. *Hum Pathol.* 2003;34:119-29.
15. Matsumoto Y, Horiike S, Ohshiro M, et al. Expression of master regulators of helper T-cell differentiation in peripheral T-cell lymphoma, not otherwise specified, by immunohistochemical analysis. *Am J Clin Pathol.* 2010;133:281-90.
16. Rijlaarsdam JU, Scheffer E, Meijer CJ, et al. Cutaneous pseudo-T-cell lymphomas. A clinicopathologic study of 20 patients. *Cancer.* 1992;69:717-24.

17. Rijlaarsdam JU, Willemze R. Cutaneous pseudolymphomas: classification and differential diagnosis. *Semin Dermatol.* 1994;13:187-96.
18. Rodriguez Pinilla SM, Roncador G, Rodriguez-Peralto JL, et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol.* 2009;33:81-90.
19. Rodriguez-Pinilla SM, Atienza L, Murillo C, et al. Peripheral T-cell lymphoma with follicular T-cell markers. *Am J Surg Pathol.* 2008;32:1787-99.
20. Samimi S, Benoit B, Evans K, et al. Increased programmed death-1 expression on CD4+ T cells in cutaneous T-cell lymphoma: implications for immune suppression. *Arch Dermatol.* 2010;146:1382-8.
21. Smolle J, Torne R, Soyer HP, et al. Immunohistochemical classification of cutaneous pseudolymphomas: delineation of distinct patterns. *J Cutan Pathol.* 1990;17:149-59.
22. Swerdlow SH, Campo E, Harris NL, et al. WHO classification of tumors - Haematopoietic and lymphoid tissues, Geneva, Switzerland. WHO PRESS. 2008.
23. Wada DA, Wilcox RA, Harrington SM, et al. Programmed death 1 is expressed in cutaneous infiltrates of mycosis fungoides and Sezary syndrome. *Am J Hematol.* 2011;86:325-7.
24. Willemze R, Jaffe ES, Burg G, et al. WHO-EORTC classification for cutaneous lymphomas. *Blood.* 2005;105:3768-85.
25. Xerri L, Chetaille B, Serriari N, et al. Programmed death 1 is a marker of angioimmunoblastic T-cell lymphoma and B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia. *Hum Pathol.* 2008;39:1050-8.
26. Yu H, Shahsafaei A, Dorfman DM. Germinal-center T-helper-cell markers PD-1 and CXCL13 are both expressed by neoplastic cells in angioimmunoblastic T-cell lymphoma. *Am J Clin Pathol.* 2009;131:33-41.
27. Yuan CM, Vergilio JA, Zhao XF, et al. CD10 and BCL6 expression in the diagnosis of angioimmunoblastic T-cell lymphoma: utility of detecting CD10+ T cells by flow cytometry. *Hum Pathol.* 2005;36:784-91.
28. Zhan HQ, Li XQ, Zhu XZ, et al. Expression of follicular helper T cell markers in nodal peripheral T cell lymphomas: a tissue microarray analysis of 162 cases. *J Clin Pathol.* 2011;64:319-24.

3

DIFFERENTIAL EXPRESSION OF PROGRAMMED DEATH-1 (PD-1) IN SÉZARY SYNDROME AND MYCOSIS FUNGOIDES

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ABSTRACT

Objective: To determine if there are differences in the expression of programmed death-1 (PD-1) between Sézary syndrome (SS) and mycosis fungoides (MF), and in particular erythrodermic MF (E-MF). PD-1 is a marker of follicular helper T (TFH) cells and is expressed by the neoplastic T cells of some types of malignant T-cell lymphoma, including SS and MF. Reported results of PD-1 staining in SS and MF are, however, conflicting.

Design: Formalin-fixed, paraffin-embedded skin biopsy specimens were stained for PD-1. In addition, PD-1⁺ cases were stained with antibodies against BCL6, CXCL13, and CD10 to find possible relationship with TFH cells.

Setting: Tertiary referral center for cutaneous lymphomas.

Patients: Twenty-seven patients with SS and 60 patients with MF, including eight patients with E-MF.

Results: In patients with SS, expression of PD-1 by more than 50% of the neoplastic T cells was observed in 24 of 27 cases (89%). In contrast, PD-1 expression by more than 50% of neoplastic T cells was found in only 8 of 60 patients with MF (13%), including only 1 of 8 patients with E-MF (12%). In PD-1⁺ cases, serial skin sections showed that CXCL13 and BCL6 generally stained 25% to 50% of the PD-1⁺ cells, while expression of CD10 was uncommon.

Conclusions: The results of the present study show differential expression of PD-1 between SS and MF/E-MF, which provides further support for the view that SS and MF are distinct entities.

INTRODUCTION

Recent studies described the expression of membrane molecule programmed death-1 (PD-1; CD279) by the neoplastic T cells of various types of malignant T-cell lymphomas, including Sézary syndrome (SS) and mycosis fungoides (MF).¹⁻⁶ PD-1 belongs to the CD28/CTLA-4 receptor family and has a key role in regulating cellular immune responses.^{7,8} PD-1 has 2 identified ligands called PD-L1 (CD274), which has a broad expression profile, including non-immune cells, and PD-L2 (CD273), which is found primarily on activated professional antigen-presenting cells.^{7,8} Engagement of PD-1 with its ligands has been shown to inhibit T-cell activation and proliferation.⁹ Since loss of PD-1 is associated with the development of autoimmune diseases, PD-1 is considered to play an important role in peripheral tolerance.^{7,8} Whereas activation is necessary to induce PD-1 expression by T cells, B cells, and dendritic cells,⁸ some cell types, such as follicular helper T (TFH) cells, have a constitutive high expression of PD-1.¹⁰ TFH cells, which also highly express chemokine (C-X-C motif) ligand 13 (CXCL13) and B-cell lymphoma 6 (BCL6), are localized in the lymph node follicles and provide essential support to germinal center formation and plasma cell development.

Recent studies showed that the neoplastic cells in angioimmunoblastic T-cell lymphoma (AITL) express PD-1, CXCL13, BCL6 and CD10.^{1,3} Because of this phenotypically resemblance to TFH cells, AITL is now commonly considered to be a tumor of TFH cells. More recently, we and others reported that the atypical medium- to large-sized CD4⁺ T cells in primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma (PCSM-TCL) also express PD-1. Since 25% to 75% of these PD-1⁺ cells also express CXCL13 and BCL6, it has been suggested that this type of cutaneous T-cell lymphoma (CTCL) may also originate from TFH cells.^{11,12}

Samimi *et al.* found increased PD-1 expression in the circulating neoplastic T cells in patients with SS, but not in CD4⁺ peripheral blood T cells from patients with MF or healthy controls, and suggested that PD-1 may contribute to immunosuppression in SS.⁵ In other studies, PD-1 expression by the neoplastic T cells was found in 50% to 60% of skin biopsy samples of patients with MF and 73% of skin biopsy samples of those with SS.^{6,13,14} In contrast, in our recent study on PCSM-TCL, PD-1 expression was detected in only 2 of 21 MF biopsy samples, included as a control group.¹² These discrepant results prompted us to investigate PD-1 expression in a large group of biopsy samples obtained from patients with SS and those with different stages of MF. In addition, we also investigated all PD-1⁺ cases for expression of BCL6, CXCL13, and CD10 to determine if these cells have a TFH cell phenotype. Our results show differential expression of PD-1 in SS and MF, which provides further support to the view that SS and MF are distinct entities.

MATERIALS AND METHODS

Patients

Paraffin-embedded skin biopsy specimens from 27 patients with SS and 60 patients with MF were selected for this study. All skin samples were collected from the archives of the Department of Pathology, Leiden University Medical Center (LUMC) according to the “Dutch Code for Proper Secondary Use of Human Tissue”, which is approved by the Medical Ethics Committee of the LUMC. In some patients, multiple skin biopsy specimens obtained from different stages of disease or during follow-up, and additional biopsy specimens from involved lymph nodes were studied. All cases had been reviewed by an expert panel of dermatologists and pathologists during one of the quarterly meetings of the Dutch Cutaneous Lymphoma group, and were classified according to the criteria of the World Health Organization – European Organization for Research and Treatment of Cancer (WHO-EORTC) for primary cutaneous lymphomas.¹⁵ Stage of disease was based on the revised International Society for Cutaneous Lymphomas/EORTC criteria.¹⁶ All patients with SS had erythroderma and peripheral blood involvement at first presentation (T4N0-3B2). Twenty-five of 27 patients with SS showed clonal T-cell receptor gene rearrangement in combination with a CD4/CD8 ratio more than 10 (range, 12 – 232) in the peripheral blood. In the other 2 patients, peripheral blood involvement was confirmed by the presence of a T-cell clone in combination with either a CD4⁺/CD7⁻ ratio greater than 40 or the presence of more than 1000 Sézary cells/mm³.

The MF group included 30 biopsy specimens from patients with patches or plaques (stage IA-IB), 22 biopsy specimens from skin tumors (19 from patients with stage IIB disease, 3 from patients with stage IV) and 8 biopsy specimens from patients with E-MF (3 patients with stage III disease; 5 patients with stage IV). Twenty-one of these 60 skin biopsy specimens were included in a previous study, where they served as a control group.¹² With respect to the E-MF group, 5 patients had a history of classical (folliculotropic) MF and developed E-MF with (cases 2 – 4) (Table 1) or without (cases 1 and 5) (Table 1) peripheral blood involvement 24 to 240 months after initial diagnosis, while 3 presented with erythroderma with the histologic features of MF, but without peripheral blood involvement (cases 6 – 8) (Table 1). Since the definition and terminology of cases with E-MF is subject to debate, the main features of these 8 cases have been summarized in Table 1.

Histologic and immunohistochemical analysis

In sections from all biopsy specimens, we routinely performed hematoxylin-eosin stainings and immunostainings to detect T-cell-associated antigens (CD2, CD3, CD4, CD5, CD8), B-cell-associated antigens (CD20 and/or CD79a), and histiocytic

Table 1. Characteristics of patients with erythrodermic mycosis fungoides (E-MF)

Patient No./ Sex/Age, y ^a	Stage	Folliculotropic MF	CD4/CD8 Ratio Blood	T-cell Clonality Blood	PD-1 Skin, %	PD-1 Lymph Node, %	Development of E-MF after Diagnosis, mo
1A/F/67	IB (T2N0B0)	–	ND	ND	ND	ND	
1B/F/74	IVA (T4N3B0)	–	2	–	–	–	96
2A/M/51	IB (T2N0B0)	+	ND	ND	>75	ND	
2B/M/71	IVA (T4N0B2)	+	35	+	>75	ND	240
3A/F/60	IB (T2N0B0)	+	ND	ND	–	ND	
3B/F/64 ^b	IVA (T4Nx B2)	+	40	+	–	ND	48
4A/M/50	IIA (T2N2B0)	+	9	ND	ND	ND	
4B/M/52	IVA (T4N2B2)	+	20	+	<10	ND	24
5A/M/58	IB (T2N0B0)	–	ND	ND	ND	ND	
5B/M/63	III (T4N3B0)	–	2	–	–	–	64
6AB/M/76 ^b	III (T4N3B0)	–	1	–	<10	–	0
7AB/M/64	III (T4N1B0)	–	13	–	–	ND	0
8AB/M/62	IVA (T4N3B0)	+	2	ND	10-15	<5	
8FU/M/63 ^b	IVA (T4N3B2)	+	12	+	10-15	ND	0

Abbreviations: FU, follow-up; ND, no data; PD-1, programmed death-1; +, positive; –, negative

^a A: at time of first diagnosis; B: at time of E-MF; AB: E-MF at presentation

^b Follow-up biopsy specimens of erythrodermatous skin were also negative for PD-1

antigens (CD68) and CD30. Sections from all patients were stained for PD-1, and in addition, PD-1⁺ cases were also stained for BCL6, CXCL13, and CD10. Staining procedures for PD-1 (1:200, goat polyclonal antibody AF1086) and CXCL13 (1:400, goat polyclonal antibody AF801), both purchased from R&D Systems (Abingdon, UK), and BCL6 (1:50, monoclonal M-7211) from Dako, (Glostrup, Denmark) have been previously described.¹² The percentage of PD-1⁺ neoplastic T cells was scored as less than 10%, 11% to 25%, 26% to 50%, and more than 50%. A case was considered PD-1⁺ when more than 50% of the neoplastic T cells were stained.

Statistic analysis

Statistical calculations were performed using SPSS statistical software (version 17.0) (SPSS Inc., Chicago, IL). Comparison of PD-1 expression between SS and MF (including E-MF) and SS and MF was performed using the Fisher exact test. All p-values were 2-tailed and p < 0.05 was considered statistically significant.

RESULTS

Sézary syndrome

Routine immunostaining revealed a CD3⁺, CD4⁺, CD8⁻ mature T-cell phenotype in all SS cases. Marker loss of either CD2, CD3, or CD5, defined as absent staining by more than 50% of the neoplastic T cells, was detected in only 1 of 27 SS cases (3.7%), in which the neoplastic cells lacked CD2. In 2 other cases there was partial loss (< 50% loss) of either CD3 or CD2. Staining for CD30 was performed in 24 of 27 cases. In 4 cases, CD30 expression was found in almost 100%, more than 50%, 30%, and 15% of the neoplastic T cells, respectively, while in the other 20 cases no or few (< 10%) CD30⁺ neoplastic T cells were found.

In 24 of the 27 SS cases, more than 50% of the neoplastic T cells in the skin expressed PD-1, in 22 cases even more than 75% (Figure 1 and Table 2). Six follow-up biopsy specimens of 3 of these 24 patients, obtained 24 to 48 months after diagnosis, also showed expression of PD-1 by more than 75% of the neoplastic T cells. PD-1 was expressed by small to large atypical T cells with cerebriform nuclei (Sézary cells) as well as by (CD30⁺) blast cells. In the other three cases, PD-1 was expressed by approximately 25% in 1 case, less than 10% in another case, while in the third case the neoplastic T cells were completely negative for PD-1. In the patient with less than 10% PD-1⁺ neoplastic T cells in the skin infiltrate, the lymph node biopsy specimen showed a diffuse population of PD-1⁺, CD3⁺, CD4⁺ neoplastic T cells. A diffuse infiltration by PD-1⁺ tumor cells was also observed in the lymph node biopsy specimens from 3 other patients with SS.

Serial skin sections showed that CXCL13 and BCL6 generally stained 25% to 50% of the PD-1⁺ cells (Figure 1). In the 2 cases with less than 10% PD-1⁺ tumor cells, CXCL13 was expressed by 25% to 50% and BCL6 by approximately 25% of the neoplastic T cells. Staining for CD10 was performed in 25 cases, and results were negative in 21 cases. In the other 4 cases, all of them with more than 50% PD-1⁺ tumor cells, CD10 was expressed by 100% of the neoplastic T cells in 1 case and by 25% to 50% in the other 3.

Mycosis fungoides

Immunostaining showed a CD4⁺CD8⁻ T-cell phenotype in 36 patients, a CD4⁻CD8⁺ T-cell phenotype in 9 patients and a CD4⁻CD8⁻ T-cell phenotype in 15 of 60 patients. Including the 15 CD4⁻CD8⁻ cases, marker loss was observed in 35 of 60 biopsy specimens (58%), including 16 of 30 patches or plaques (53%), 14 of 22 tumors (64%), and 5 of 8 E-MF (63%). Expression of CD30 by more than 25% of the neoplastic T cells was observed in 11 biopsy specimens, including 5 plaques, 5 tumors and 1 biopsy obtained from E-MF.

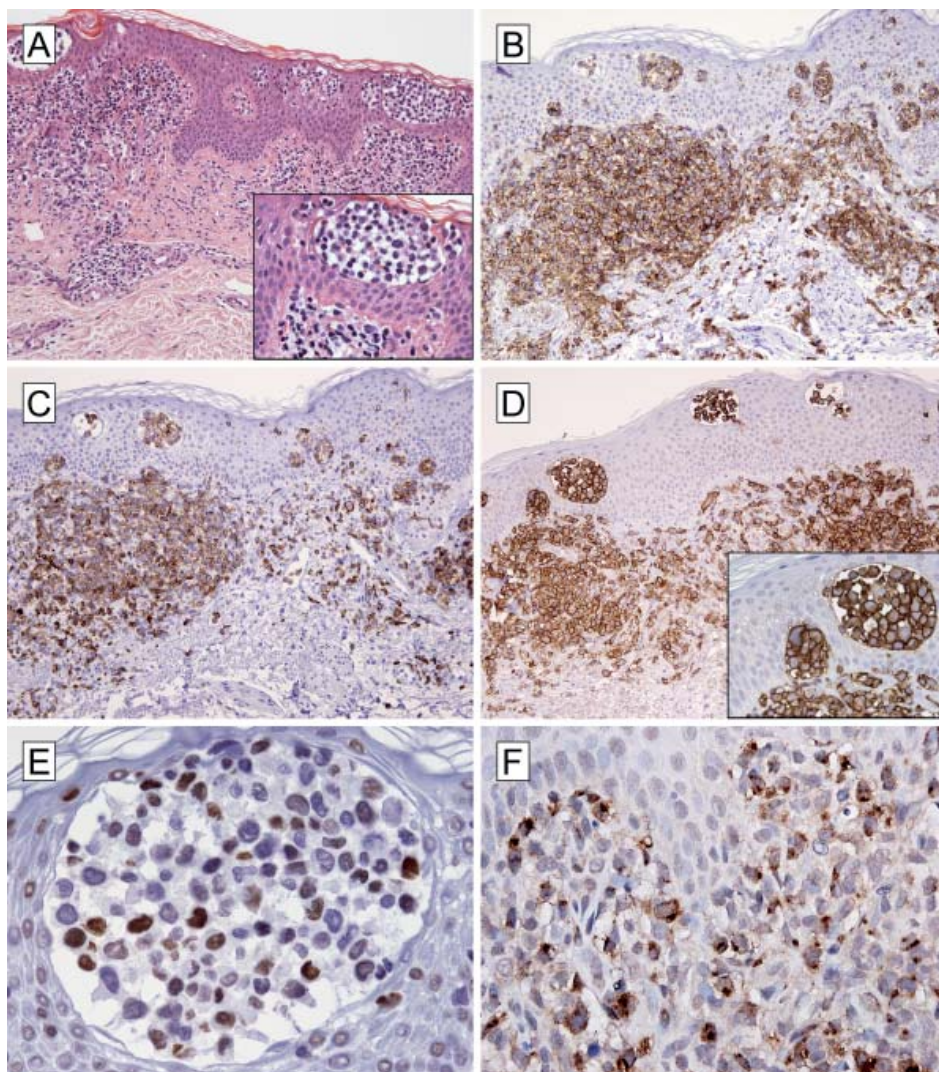


Figure 1. Histopathologic features of a representative patient with Sézary syndrome. (A) The hematoxylin-eosin staining of the lesion showed a superficial band-like dermal infiltrate and epidermal Pautrier microabscesses with CD4+ (B) CD3+ (C) neoplastic T cells. These neoplastic T cells showed strong PD-1 positivity (D), and serial skin sections showed that BCL6 (E) and CXCL13 (F) was expressed by 25% to 50% of the PD-1+ cells. A-D, Original magnification x100. E and F, Original magnification x400. Insets in A and D, original magnification x200.

Expression of PD-1 by more than 50% of the neoplastic T cells was observed in 8 of 60 patients (13%), including 4 of 30 patches or plaques (13%), 3 of 22 tumors (14%), and interestingly, only 1 of 8 biopsy specimens from E-MF (13%). PD-1 expression was significantly higher in the SS group compared with the total MF

Table 2. Results of PD-1 expression in skin biopsy specimens of patients with SS and MF

Type of lymphoma	Cases, No.	PD-1 ⁺ neoplastic T cells, No. (%)		
		>50%	11%-50%	<10%
SS	27 ^{a,b}	24 (89%)	1 (4%)	2 (7%)
MF	60 ^a	8 (13%)	4 (7%)	48 (80%)
Patch/plaque	30	4 (13%)	2 (7%)	24 (80%)
Tumor	22	3 (14%)	1 (4%)	18 (82%)
Erythrodermic MF	8 ^b	1 (12%)	1 (12%)	6 (75%)

^a Patients with SS (n=27) versus those with MF (n=60): $p < 0.001$

^b Patients with SS (n=27) versus those with erythrodermic MF (n=8): $p < 0.001$

group ($p < 0.001$), as well as compared with the E-MF group ($p < 0.001$). The results of PD-1 staining in a second biopsy, obtained from either a preceding plaque or a subsequent tumor in 8 of 60 patients, were identical to those of the first biopsy. The 8 PD-1⁺ cases included both patients with a CD4⁺CD8⁻ phenotype (4 cases), a CD4⁺CD8⁺ phenotype (1 case) and a CD4⁺CD8⁻ T-cell phenotype (3 cases). In these cases, PD-1 stained both atypical T cells with cerebriform nuclei and (CD30⁺) blast cells. In the 11 biopsy specimens containing more than 25% CD30⁺ tumor cells, PD-1 was expressed by 30% to more than 75% of the CD30⁺ blast cells in 3 cases, while these cells were negative for PD-1 in the other 8 cases. In addition to the 8 cases with more than 50% PD-1⁺ tumor cells, in 1 case 25% to 50% and in 3 cases 11% to 25% of the neoplastic T cells expressed PD-1 (Table 2). In the other 48 cases, no or sometimes few (but always < 10%) neoplastic T cells expressed PD-1 (Figure 2), while in all biopsy specimens small numbers of reactive PD-1⁺ T cells were observed, serving as an internal control. Examination of serial sections showed that both CXCL13 and BCL6 generally stained 25% to 50% of the PD-1⁺ cells. CD10 was expressed by almost all neoplastic T cells in only 1 of 8 biopsy specimens with more than 50% PD-1⁺ tumor cells, and in none of the 10 biopsy specimens with 10% to 50% PD-1⁺ tumor cells.

Additional studies in E-MF

After this initial analysis additional skin and lymph node biopsy specimens were collected from 6 of 8 patients with E-MF. These biopsies were obtained from plaques 4 and 20 years before the development of erythroderma (cases 2 and 3) (Table 1), from erythrodermatous skin during follow-up 12 to 60 months after diagnosis (cases 3, 6 and 8) (Table 1), and from diffusely involved lymph nodes (N3 stage; cases 1, 5, 6, and 8) (Table 1). Four patients showed folliculotropic infiltrates both in the first biopsy specimens at the nonerythrodermatic stage and in the

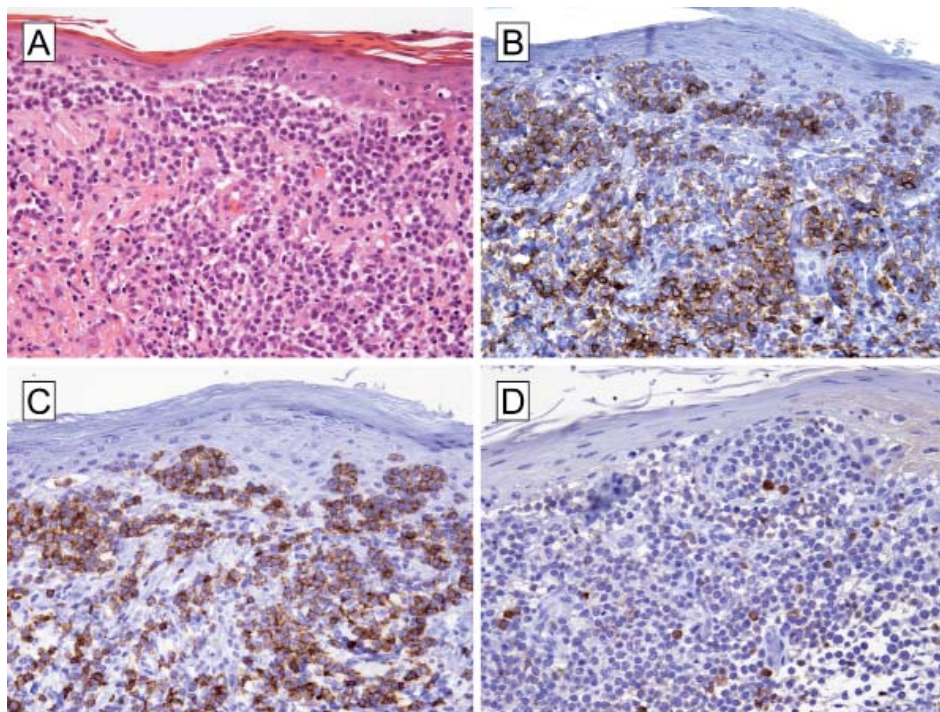


Figure 2. Histopathologic features of a representative patient with MF who was negative for programmed death-1 (PD-1). (A) The hematoxylin-eosin staining (original magnification x100) of the lesion showed an epidermal and superficial dermal infiltrate with CD4+ (B) CD3+ (C) neoplastic T cells. PD-1 expression was seen in less than 10% of the tumor cells (D).

follow-up biopsy specimens at the erythrodermatic stage. In the single patient who was PD-1⁺ (case 2) (Table 1), PD-1 was expressed by more than 75% of the neoplastic T cells both in the erythrodermatous skin and in the plaque biopsied 20 years before. In the 5 other patients, PD-1 was consistently negative, both in preceding plaques, follow-up biopsy specimens and involved lymph nodes (Table 1).

DISCUSSION

In the present study we observed a differential expression of PD-1 in skin biopsy specimens from patients with SS and MF. In 24 of 27 patients with SS (89%), PD-1 was expressed by more than 50% of the malignant T cells, while in 1 of the 3 remaining cases the neoplastic cells in the involved lymph node were PD-1⁺. In contrast, in only 8 of 60 patients with MF (13%), PD-1 was expressed by more than 50% of the neoplastic cells.

The proportion of PD-1⁺ MF cases (13%) in our study contrasts with the results of 3 other studies.^{6,13,14} Wada et al.⁶ reported a percentage of more than 50% PD-1⁺ tumor cells in 6 of 15 patches or plaques (40%), 9 of 15 tumors in MF (60%), and in 8 of 11 biopsy specimens from patients with SS (63%). In the study of Kantekure et al.¹³ 16 of 26 MF biopsy specimens (62%) contained more than 25% PD-1⁺ tumor cells, but if the cut-off point of 50% of the present study was used, only 7 of 26 biopsy specimens from patients with MF (26%) were PD-1⁺. Roncador et al.¹⁴ observed PD-1 expression in 5 of 9 MF cases (56%) but did not provide a cut-off point for PD-1 positivity. The results of the present study are consistent with those of a previous study of our group.¹² In that study, which was focused on PD-1 expression in PCSM-TCL and/or pseudo-T-cell lymphomas, 21 cases of MF with a CD4⁺CD8⁻ T-cell phenotype were included as a control group, and expression of PD-1 by more than 50% of the neoplastic T cells was found in only 2 of 21 cases. In the present study, we did not only include cases with a CD4⁺ T-cell phenotype, but—to cover the whole spectrum of MF—our database was actively searched for cases with a CD4⁺CD8⁺ and CD4⁺CD8⁻ T-cell phenotype, cases with CD30 expression in a considerable proportion of tumor cells (since 1 of the 2 PD-1⁺ MF cases from our previous study strongly expressed CD30), as well as cases with E-MF. PD-1 expression was found not only in cases with a CD4⁺CD8⁻ T-cell phenotype, but also in cases with a CD4⁺CD8⁺ or CD4⁺CD8⁻ T-cell phenotype, and both in CD30⁺ and CD30⁻ cases. The expression of PD-1 by CD30⁺ blast cells in some cases of MF and SS is of interest, since the neoplastic CD30⁺ T cells in cutaneous anaplastic large-cell lymphoma and lymphomatoid papulosis are consistently negative for PD-1.¹²

Remarkably, expression of PD-1 by more than 50% of the neoplastic T cells was observed in only 1 of the 8 patients with E-MF. In this patient, both erythroderma and the initial plaque-like lesion biopsied 20 years before were strongly positive for PD-1. In the other 7 patients, all skin and lymph node biopsy specimens were consistently negative for PD-1. It should be noted that, during follow-up, 4 patients in this group developed peripheral blood involvement, meeting the criteria normally used for the diagnosis of SS (B2 stage) (Table 1). There is controversy as to how such patients with MF developing peripheral blood involvement during follow-up should be designated. Some might prefer to simply use the term SS,¹⁷ others like SS *preceded by MF*¹⁸ or *secondary SS*,¹⁹ but we designate such cases *E-MF with blood involvement*.

These observations are important for the ongoing discussion regarding the relationship between SS and MF. Often, SS is designated as a leukemic phase or leukemic variant of MF. Indeed, there are morphological (atypical cells with cerebriform nuclei) and phenotypical (CD3⁺CD4⁺CD8⁻ T cells) similarities between both conditions, which resulted in the concept of CTCL.²⁰ Moreover, patients with MF may sometimes present with erythroderma and/or develop peripheral blood involvement during follow-up, while rare patients with SS may develop skin

tumors as in MF. However, apart from the differences in clinical presentation and clinical course, there are also histological, phenotypical and genetic differences between both conditions. Histologically, the neoplastic T cells in early-stage MF preferentially colonize the basal layers of the epidermis, while the neoplastic T cells in SS show a preferential perivascular distribution with or without prominent epidermal infiltration, reflecting the leukemic nature of the disease.^{19;20} Consistently, involved lymph nodes in SS are generally overrun by a monotonous infiltration of Sézary cells.²¹ In contrast, involved lymph nodes in MF show increased numbers of both atypical T cells with cerebriform nuclei and interdigitating reticulum cells, and in advanced stages blast cell transformation is much more pronounced than in SS lymph nodes.²¹ Phenotypically, SS consistently has a mature CD4⁺ T-cell phenotype. Also in MF, a CD4⁺ phenotype is most common, but patients may also present with a CD4-CD8⁺ or a CD4-CD8⁻ T-cell phenotype.²² Moreover, both in the present study and previous studies on involved lymph nodes, loss of pan-T-cell antigens (CD2, CD3, CD5) is frequently observed in skin lesions and involved lymph nodes in MF, in particular in patients with advanced disease.²³ In contrast, the neoplastic T cells in skin and lymph nodes of patients with SS generally retain their mature CD4⁺ T-cell phenotype, even in patients with advanced disease.²³ In the present study, loss of pan-T-cell antigens was observed in only 1 of 27 patients with SS. More recent phenotypical studies showed that the neoplastic T cells obtained from the peripheral blood of patients with SS have a central memory T-cell phenotype, while T cells isolated from MF skin lesions had skin resident effector memory T-cell phenotype.^{24;25} The authors suggested that SS and MF should be considered as separate lymphomas arising from distinct functional T-cell subsets.^{24;25} Also, recent genetic studies showing major oncogenetic differences between SS and tumor-stage MF suggested that these are distinct entities.^{26;27} The almost consistent expression of PD-1 and CXCL13 in SS²⁸ and the uncommon expression in MF also support the view that SS and MF should be considered as distinct entities. This would imply that in future trials these conditions should be considered separately.

The consistent expression of PD-1, CXCL13, and BCL6 by the neoplastic cells in SS is similar to that of TFH cells in the germinal centers of lymph nodes, and similar to the neoplastic T cells in AITL, now commonly designated as a tumor of TFH cells.^{1;3} This raises the question of whether the neoplastic cells in SS are derived from TFH cells and/or have the functional characteristics of these TFH cells. TFH cells are located in the germinal centers in lymph nodes providing essential support for B-cell maturation and plasma cell development. The chemokine CXCL13 selectively recruits CXCR5⁺ B cells into the follicle and is involved in the activation of B cells.¹⁰ CXCL13 is expressed by the malignant T cells in lymph nodes in AITL and has been suggested to contribute to B-cell dysregulation.²⁹ However, such a role seems unlikely in SS. Admixture of B cells is uncommon in skin biopsy specimens of SS,

and also in our MF biopsy specimens no relationship between PD-1 expression and the number of admixed B cells or plasma cells was found (data not shown).

Two recent studies suggest another role for CXCL13 and PD-1 in SS. Picchio *et al.* found high expression of CXCL13 in skin lesions, lymph nodes and peripheral blood of patients with SS and noted that plasma levels of functional active CXCL13 increased significantly during disease progression.²⁸ Interestingly, while the neoplastic T cells in most patients with SS were positive for CXCL13 (13 of 16 [81%] cases), in patients with MF only 4 of 14 cases (28%) expressed CXCL13, which nicely parallels our observations on PD-1 expression in these conditions. Picchio *et al.*²⁸ proposed that CXCL13 acts in synergy with CCR7 agonists CCL21 (present in both skin and lymph node of patients with SS) to promote the skin and lymph node homing of the Sézary cells that are known to have high expression CCR7.³⁰ In another study, Samimi *et al.*⁵ found increased PD-1 expression in the circulating neoplastic T cells in patients with SS compared with patients with MF and healthy controls. They observed a strong decrease in PD-1 expression with improvement of disease. Moreover, an increase in interferon gamma production was found in cultures of PBMC from patients with SS if the PD-1/PD-L1 interaction was interfered by blocking anti-PD-1 antibodies. They suggested that the high PD-1 expression may contribute to immunosuppression in SS, but the mechanisms involved are as yet unknown. Given the high expression of PD-1 in Sézary cells, it is tempting to speculate that PD-1 on Sézary cells may serve as a potential target for immunotherapy. Interestingly, fully-human blocking antibodies for PD-1 have recently been tested in a phase I clinical trial, showing promising results for different types of PD-L1⁺ solid tumors that are assumed to attenuate the PD-1⁺ tumor infiltrating T lymphocytes.³¹ Although in case of SS the situation is different—SS is a T-cell tumor and the neoplastic cells are PD-1⁺ themselves—it may very well be that patients with SS may benefit from anti-PD-1 immunotherapy as well.

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REFERENCE LIST

1. Rodriguez-Pinilla SM, Atienza L, Murillo C et al. Peripheral T-cell lymphoma with follicular T-cell markers. *Am J Surg Pathol* 2008;32:1787-1799.
2. Yu H, Shahsafaei A, Dorfman DM. Germinal-center T-helper-cell markers PD-1 and CXCL13 are both expressed by neoplastic cells in angioimmunoblastic T-cell lymphoma. *Am J Clin Pathol* 2009;131:33-41.
3. Zhan HQ, Li XQ, Zhu XZ, Lu HF, Zhou XY, Chen Y. Expression of follicular helper T cell markers in nodal peripheral T cell lymphomas: a tissue microarray analysis of 162 cases. *J Clin Pathol* 2011;64:319-324.
4. Yuan CM, Vergilio JA, Zhao XF, Smith TK, Harris NL, Bagg A. CD10 and BCL6 expression in the diagnosis of angioimmunoblastic T-cell lymphoma: utility of detecting CD10+ T cells by flow cytometry. *Hum Pathol* 2005;36:784-791.
5. Samimi S, Benoit B, Evans K et al. Increased programmed death-1 expression on CD4+ T cells in cutaneous T-cell lymphoma: implications for immune suppression. *Arch Dermatol* 2010;146:1382-1388.
6. Wada DA, Wilcox RA, Harrington SM, Kwon ED, Ansell SM, Comfere NI. Programmed death 1 is expressed in cutaneous infiltrates of mycosis fungoides and Sezary syndrome. *Am J Hematol* 2011;86:325-327.
7. Fife BT, Pauken KE. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Ann N Y Acad Sci* 2011;1217:45-59.
8. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 2010;236:219-242.
9. Jin HT, Ahmed R, Okazaki T. Role of PD-1 in regulating T-cell immunity. *Curr Top Microbiol Immunol* 2011;350:17-37.
10. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011;29:621-663.
11. Rodriguez Pinilla SM, Roncador G, Rodriguez-Peralto JL et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol* 2009;33:81-90.
12. Cetinozman F, Jansen PM, Willemze R. Expression of Programmed Death-1 in Primary Cutaneous CD4-Positive Small/Medium-Sized Pleomorphic T-Cell Lymphoma, Cutaneous Pseudo-T-Cell Lymphoma, and Other Types of Cutaneous T-Cell Lymphoma. *Am J Surg Pathol* 2012;36:109-116.
13. Kantekure K, Yang Y, Raghunath P et al. Expression Patterns of the Immunosuppressive Proteins PD-1/CD279 and PD-L1/CD274 at Different Stages of Cutaneous T-Cell Lymphoma/Mycosis Fungoides. *Am J Dermatopathol* 2011.
14. Roncador G, Garcia Verdes-Montenegro JF, Tedoldi S et al. Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. *Haematologica* 2007;92:1059-1066.
15. Willemze R, Jaffe ES, Burg G et al. WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005;105:3768-3785.
16. Olsen E, Vonderheid E, Pimpinelli N et al. Revisions to the staging and classification of mycosis fungoides and Sezary syndrome: a proposal of the International Society for

- Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). *Blood* 2007;110:1713-1722.
17. Agar N, Whittaker SJ. Folliculotropic mycosis fungoides (stage IIA) progressing to Sezary syndrome: a case report. *Br J Dermatol* 2008;159:1197-1199.
 18. Vonderheid EC, Bernengo MG, Burg G et al. Update on erythrodermic cutaneous T-cell lymphoma: report of the International Society for Cutaneous Lymphomas. *J Am Acad Dermatol* 2002;46:95-106.
 19. Diwan AH, Prieto VG, Herling M, Duvic M, Jone D. Primary Sezary syndrome commonly shows low-grade cytologic atypia and an absence of epidermotropism. *Am J Clin Pathol* 2005;123:510-515.
 20. Sentis HJ, Willemze R, Scheffer E. Histopathologic studies in Sezary syndrome and erythrodermic mycosis fungoides: a comparison with benign forms of erythroderma. *J Am Acad Dermatol* 1986;15:1217-1226.
 21. Scheffer E, Meijer CJ, van Vloten WA, Willemze R. A histologic study of lymph nodes from patients with the Sezary syndrome. *Cancer* 1986;57:2375-2380.
 22. Massone C, Crisman G, Kerl H, Cerroni L. The prognosis of early mycosis fungoides is not influenced by phenotype and T-cell clonality. *Br J Dermatol* 2008;159:881-886.
 23. Willemze R, Scheffer E, Meijer CJ. Immunohistochemical studies using monoclonal antibodies on lymph nodes from patients with mycosis fungoides and Sezary's syndrome. *Am J Pathol* 1985;120:46-54.
 24. Campbell JJ, Clark RA, Watanabe R, Kupper TS. Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. *Blood* 2010;116:767-771.
 25. Clark RA, Watanabe R, Teague JE et al. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Sci Transl Med* 2012;4:117ra7.
 26. van Doorn R., van Kester MS, Dijkman R et al. Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. *Blood* 2009;113:127-136.
 27. Booken N, Gratchev A, Utikal J et al. Sezary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNM3. *Leukemia* 2008;22:393-399.
 28. Picchio MC, Scala E, Pomponi D et al. CXCL13 is highly produced by Sezary cells and enhances their migratory ability via a synergistic mechanism involving CCL19 and CCL21 chemokines. *Cancer Res* 2008;68:7137-7146.
 29. Balaraman B, Conley JA, Sheinbein DM. Evaluation of cutaneous angioimmunoblastic T-cell lymphoma. *J Am Acad Dermatol* 2011;65:855-862.
 30. Sokolowska-Wojdylo M, Wenzel J, Gaffal E et al. Circulating clonal CLA(+) and CD4(+) T cells in Sezary syndrome express the skin-homing chemokine receptors CCR4 and CCR10 as well as the lymph node-homing chemokine receptor CCR7. *Br J Dermatol* 2005;152:258-264.
 31. Brahmer JR, Drake CG, Wollner I et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 2010;28:3167-3175.

4

EXPRESSION OF PROGRAMMED DEATH-1 IN SKIN BIOPSIES OF BENIGN INFLAMMATORY VERSUS LYMPHOMATOUS ERYTHRODERMA

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ABSTRACT

Background: Histologic differentiation between Sézary syndrome (SS) and erythrodermic inflammatory dermatoses (EID) can be very difficult. Recent studies show that programmed death-1 (PD-1) is strongly expressed by the neoplastic cells in skin biopsies of SS, while similar studies in EID are lacking.

Objective: To determine whether the number and distribution of PD-1⁺ T cells could be used as an adjunct in the differentiation between SS and EID.

Methods: Expression of PD-1 and a panel of T-cell markers was investigated in skin biopsies from 30 patients with various types of EID (12 idiopathic, 10 atopic, 6 psoriatic and 2 paraneoplastic) and 25 patients with SS.

Results: Expression of PD-1 by more than 50% of the infiltrating T cells was observed in 23 of 25 SS cases (92%) and only in 4 of 30 EID cases (13%). PD-1 is expressed by neoplastic CD4⁺ T cells in SS, while in contrast, PD-1 was predominantly expressed by dermal and epidermal CD8⁺ T cells in EID. Expression of CD7 by $\leq 20\%$ of the infiltrating T cells was observed only in SS (13 of 24; 54%), and not in any of the 30 cases of EID.

Conclusions: While PD-1 is expressed by CD4⁺ neoplastic T cells in SS, our results suggest that PD-1 is expressed mainly by activated dermal and epidermal CD8⁺ T cells in EID. Expression of PD-1 by $> 50\%$ of CD4⁺ T cells and expression of CD7 by $\leq 20\%$ of the infiltrating T cells strongly support a diagnosis of SS in skin biopsies of patients with erythroderma.

What is already known about this topic?

- Histological differentiation between Sézary syndrome (SS) and erythrodermic inflammatory dermatoses (EID) is often difficult.
- Programmed death-1 (PD-1) is strongly expressed by the neoplastic cells in skin biopsies of SS suggesting diagnostic potential, but similar studies in EID are lacking.

What does this study add?

- PD-1 is expressed not only by CD4⁺ neoplastic T cells in SS, but also by activated (epi)dermal CD8⁺ T cells in EID.
- Expression of PD-1 by $> 50\%$ of CD4⁺ T cells and expression of CD7 by $\leq 20\%$ of the infiltrating T cells strongly support a diagnosis of SS.

INTRODUCTION

Programmed death-1 (PD-1; CD279) is expressed by activated T cells and upon binding to its ligands PD-L1 or PD-L2, a co-inhibitory signal is provided promoting apoptosis, anergy and functional exhaustion.¹ PD-1 is constitutively expressed by CD4⁺ follicular helper T (TFH) cells and plays a role in germinal center formation and plasma-cell development.² Recent studies show that the neoplastic cells in angioimmunoblastic T-cell lymphoma (AITL) express PD-1, CXCL13, BCL6 and CD10.^{3,4} Because of this phenotypical resemblance to TFH, AITL is now commonly considered as a tumor of TFH cells. We and others reported that the atypical medium-to-large CD4⁺ T cells in primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma (PCSM-TCL) also express PD-1. As 25% to 75% of these PD-1-positive cells also express CXCL13 and BCL6, it has been suggested that this type of cutaneous T-cell lymphoma (CTCL) may also originate from TFH cells.⁵⁻⁷

In another study of our group, it was shown that PD-1 was —almost without exception— strongly expressed by more than 50% of the neoplastic T cells in Sézary syndrome (SS), but uncommonly (13%) by the tumor cells in skin lesions of mycosis fungoides (MF).⁸ In other reports, PD-1 expression by the neoplastic T cells was found in 50% to 60% of skin biopsy samples of patients with MF,^{3,9,10} however, in these studies different cut-off points for PD-1 positivity were applied.⁸ Based on our previous results it is therefore tempting to consider PD-1 expression by more than 50% of the T cells in a skin biopsy of a patient with erythroderma as supportive evidence for a diagnosis of SS. However, caution is warranted, since reports on PD-1 expression in skin biopsies of patients with an erythrodermic inflammatory dermatosis (EID) are lacking. It is well known that histologic differentiation between SS and EID can be extremely difficult. In a recent study, blind evaluation of hematoxylin-eosin stained sections from skin biopsies of 18 patients with a CTCL, including 14 patients with SS and 29 patients with an EID, correct differentiation between CTCL and EID was made in approximately 50% of cases.¹¹

In the present study, the expression of PD-1 and other markers was investigated in skin biopsies of 30 patients with various types of EID, and compared with a group of 25 patients with SS described previously.⁸ In addition, the density and cellular composition of the dermal infiltrates, the extent and phenotype of epidermotropic T cells and the presence of marker loss by the infiltrating T cells were evaluated. The main goal was to investigate whether the number and distribution of PD-1⁺ T cells could be used as an adjunct in the differentiation between SS and EID.

PATIENTS AND METHODS

Patients

Paraffin-embedded skin biopsies from 30 patients with an EID were selected for this study. This group included 12 patients idiopathic erythroderma, 10 patients with atopic erythroderma, 6 patients with erythrodermic psoriasis and 2 patients with paraneoplastic erythroderma. In each patient the diagnosis was based on a combination of clinical and histological criteria, supplemented by immunophenotyping and clonality analysis of peripheral blood to exclude peripheral blood involvement by a CTCL. Review of clinical records revealed that none of the patients developed a lymphoma during follow-up. The 30 patients with an EID were compared with a group of 25 patients with SS from a previous study.⁸ The diagnosis of SS was based on the criteria of the World Health Organization–European Organization of Research and Treatment of Cancer classification (WHO–EORTC) classification of primary cutaneous lymphomas.¹² The study complied with the Declaration of Helsinki and was performed in accordance with the Dutch Code and Leiden University Medical Center guidelines on leftover material.

Histology and Immunohistochemistry

Sections from all biopsies had routinely been stained with hematoxylin/eosin and with monoclonal antibodies against T-cell-associated antigens (CD2, CD3, CD4, CD5, CD7, CD8), B-cell-associated antigens (CD20 and/or CD79a), and CD68 and CD1a to differentiate between CD4⁺ T cells and CD4⁺ histiocytes and Langerhans cells/dendritic cells, respectively. For the purpose of this study, sections from all patients were stained for PD-1, BCL6 and CXCL13, as described previously.^{6,8} Using a semiquantitative analysis on serial sections, the percentages of CD3⁺ T cells expressing CD4, CD8, PD-1, BCL6 or CXCL13 were scored as < 10%, 11–25%, 26–50% or > 50%, both in the epidermal and dermal compartments. These percentages were estimated independently by three observers (F.C., P.M.J., R.W.). In the few cases in which there was disagreement, sections were read jointly and consensus was reached. Loss of pan-T-cell antigens (CD2, CD3, CD5, CD7) was defined as expression by < 50% of CD3⁺ T cells.

RESULTS

Erythrodermic inflammatory dermatoses

The EID generally showed sparse-to-moderately dense perivascular-to-band-like infiltrates in the superficial dermis. Intraepidermal T cells were few or absent, and in some cases could only be recognized in immunostained sections. Moderate-

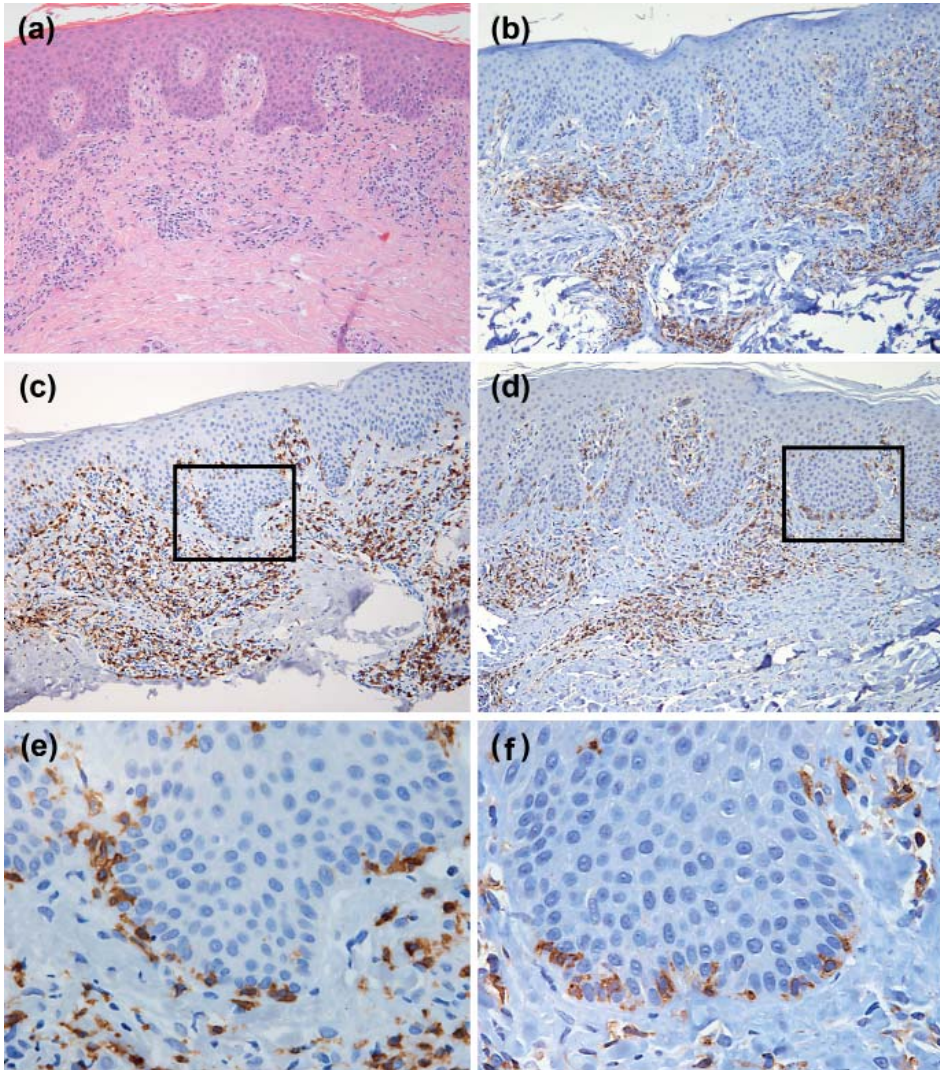


Figure 1. Skin biopsy of a patient with paraneoplastic erythroderma. (a) Haematoxylin-eosin staining of the lesional skin. (b–d) Serial sections from the same area stained for CD4 (b), CD8 (c) and programmed death-1 (PD-1) (d). Positive-stained cells can be recognized by the brown color. (e, f) Higher magnification of the marked areas in (c) and (d), respectively, showing a remarkable lining up of the CD8⁺ and PD-1⁺ intraepidermal T cells along the basal membrane. Original magnification: (a–d) x100; (e, f) x400.

to-extensive exocytosis was observed in only 4 of 30 cases (Table 1). Remarkably, in one case of paraneoplastic erythroderma, intraepidermal T cells were lined up along the basal epidermal layer (Figure 1). Pautrier microabscesses were not observed.

Table 1. Immunohistochemical results

	Idiopathic erythroderma (n = 12), n (%)	Atopic erythroderma (n = 10), n (%)	Psoriatic erythroderma (n = 6), n (%)	Paraneoplastic erythroderma (n = 2), n (%)	Sézary syndrome (n = 25), n (%)
Exocytose					
No	2 (17)	1 (10)	0	0	6 (24)
Few	8 (66)	8 (80)	6 (100)	1 (50)	8 (32)
Moderate	1 (8)	1 (10)	0	1 (50)	0
Extensive	1 (8)	0	0	0	11 (44) *
Pautrier	0	0	0	0	10 (40)
Epidermal CD4/CD8 T-cell ratio					
CD4 > CD8	2 (20)	0	0	0	14 (74)
CD4 = CD8	1 (10)	0	0	0	1 (5) †
CD4 < CD8	7 (70)	8 (100)	6 (100)	2 (100)	4 (21) †
Density dermal infiltrate					
Sparse	7 (58)	6 (60)	5 (83)	1 (50)	1 (4)
Moderate	4 (33)	4 (40)	1 (17)	1 (50)	19 (76)
Extensive	1 (8)	0	0	0	5 (20)
Dermal CD4/CD8 T-cell ratio					
CD4 > CD8	10 (83)	3 (30)	2 (33)	0	25 (100)
CD4 = CD8	2 (17)	3 (30)	3 (50)	0	0
CD4 < CD8	0	4 (40)	1 (17)	2 (100)	0
Dermal CD8⁺ T cells					
< 10%	3 (25)	0	0	0	11 (44)
10 – 25%	5 (41)	1 (10)	2 (33)	0	13 (52)
26 – 50%	4 (33)	5 (50)	3 (50)	0	1 (4)
> 50%	0	4 (40)	1 (17)	2 (100)	0
Median (range), %	20 (5–50)	50 (25–75)	30 (20–70)	67 (60–75)	12 (<5–30)
Dermal PD-1⁺ T cells					
< 10%	7 (58)	0	1 (17)	1 (50)	1 (4)
10 – 25%	5 (41)	2 (20)	2 (33)	0	1 (4)
26 – 50%	0	5 (50)	3 (50)	0	0
> 50%	0	3 (30)	0	1 (50)	23 (92)
Median (range), %	5 (<5–20)	30 (20–75)	20 (10–30)	30 (5–60)	90 (<10–100)
Loss pan-T-cell marker (> 50%)					
CD2, CD3, CD5	–	–	–	–	1 (4)
CD7	3 (25)	1 (10)	0	0	16/24 (66)

PD-1, programmed death-1. *Presence of Pautrier microabscesses is considered as extensive irrespective of the number of abscesses; † very few morphologically non-neoplastic T cells

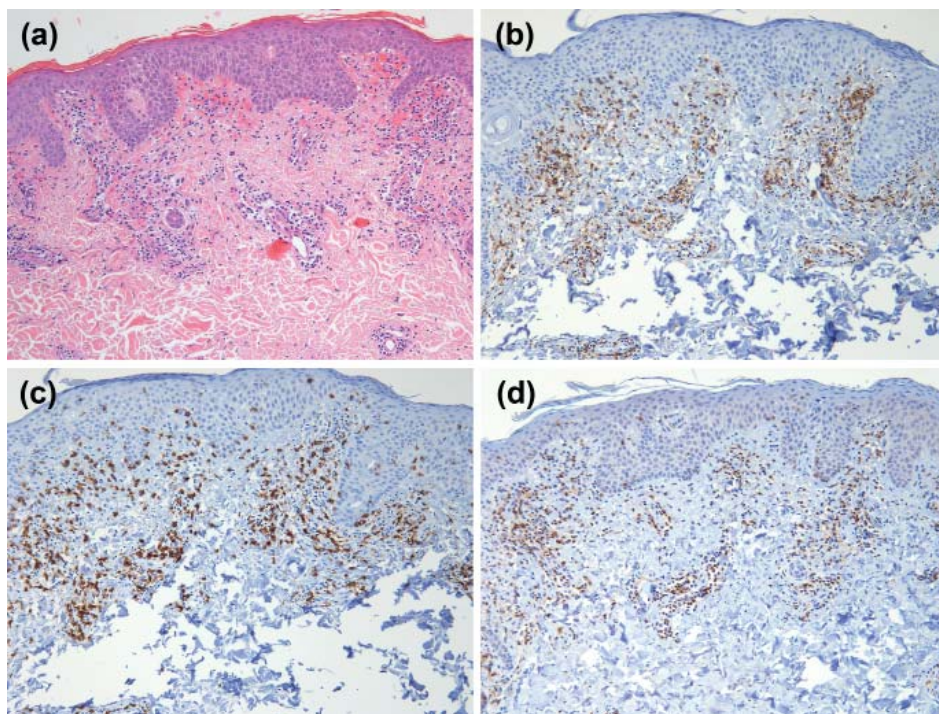


Figure 2. Skin biopsy of a patient with atopic erythroderma. (a) Haematoxylin-eosin staining of the lesion showing perivascular infiltrates. (b–d) Serial sections from the same area stained for CD4 (b), CD8 (c) and programmed death-1 (d). Original magnification x100.

The superficial dermal infiltrates were composed mainly of small lymphocytes admixed with variable numbers of histiocytes. Eosinophils were observed in 9 of 12 cases of idiopathic erythroderma, 6 of 10 cases of atopic erythroderma and one case of paraneoplastic erythroderma. In three cases of atopic erythroderma the dense superficial infiltrates showed a predominance of slightly atypical small/medium-sized pleomorphic T cells and scattered blast cells, and a diagnosis of (highly) suspected CTCL had initially been considered. One case of idiopathic erythroderma and one case of atopic erythroderma contained considerable numbers of CD30⁺ cells, while in all other cases scattered CD30⁺ blast cells were few or absent. Percentages of CD8⁺ T cells in the dermal infiltrates varied between 5% and 75% (median, 30%) of the dermal CD3⁺ T cells, and were most numerous in atopic erythroderma (Table 1). A predominance of CD8⁺ T cells (CD8/CD3 ratio > 50%) was found in 4 of 10 cases of atopic erythroderma, one of 6 cases of psoriatic erythroderma and both cases of paraneoplastic erythroderma. Percentages < 25% were found in 11 of 30 cases, including eight cases of idiopathic erythroderma (Table 1).

Although partial loss of CD7 by the dermal T cells was quite common, loss by $> 50\%$ was found in only 4 of 30 cases of EID. Loss of CD2, CD3 or CD5 was not observed. PD-1 $^{+}$ T cells varied between $< 5\%$ to 75% of the dermal CD3 $^{+}$ T cells and were most frequent in atopic erythroderma (Table 1). Percentages $> 50\%$ PD-1 positivity — used as the cut-off point for positivity in CTCL, as described before⁸ — were found in only 4 of 30 cases of EID: 3 cases of atopic erythroderma and 1 case of paraneoplastic erythroderma. Examination of serial sections suggested that in 3 of these 4 cases PD-1 was predominantly expressed by CD8 $^{+}$ dermal T cells (Figure 2). BCL6 was expressed by $< 5\%$ up to 20% (median, 10%) of the dermal T cells, while CXCL13 $^{+}$ cells were few or absent. Epidermal PD-1 $^{+}$ T cells were few or absent. If present, they generally followed the staining pattern of intraepidermal CD8 $^{+}$ T cells (Figures 1 and 2). BCL6 expression by intraepidermal T cells could not be scored, because of the strong BCL6 expression by epidermal keratinocytes. Staining for CXCL13 was generally negative.

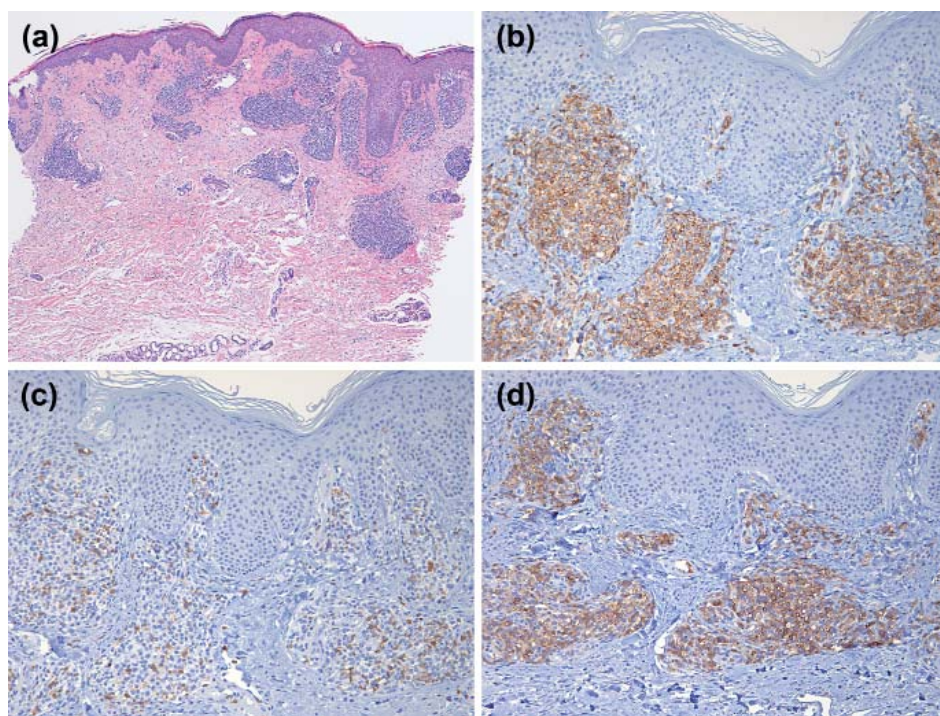


Figure 3. Skin biopsy of a patient with Sézary syndrome. (a) Haematoxylin-eosin staining of the lesion showing dense perivascular infiltrates in the papillary dermis. (b) T cells are visualized by CD3 staining. (c) CD7 expression was expressed by $< 20\%$ of the neoplastic T cells, while (d) programmed death-1 was expressed by $> 75\%$ of the neoplastic cells. Original magnification: (a) x25; (b–d) x100.

Sézary syndrome

Skin biopsies from patients with SS, investigated for PD-1 expression in a previous study⁸, characteristically showed perivascular-to-band-like infiltrates in the papillary dermis, which were generally more pronounced than observed in EID. More diffuse infiltrates extending into the reticular dermis were observed in four cases. Epidermotropic neoplastic CD4⁺ T cells were contained mainly within Pautrier microabscesses (10 cases). Extensive epidermotropism outside Pautrier microabscesses was observed in only two cases, one of them in combination with Pautrier microabscesses. In the other 14 cases no (6 cases) or only few scattered intraepidermal T cells (8 cases) were observed. The dermal infiltrates were composed predominantly of small-to-large atypical CD4⁺ T cells with hyperconvoluted nuclei (Sézary cells) and variable numbers of blast cells. In one of 25 cases, cellular atypia was minimal and a diagnosis of CTCL was at most suspected. In four cases 15% to 100% of the neoplastic T cells expressed CD30, while in the other 20 cases no or few (< 10%) CD30⁺ neoplastic T cells were found. Loss of CD7 by > 50% of the neoplastic T cells was found in 16 of 24 evaluable cases (66%), in 13 of these cases (54%) a loss of even more than 80% was observed (Figure 3). Loss of CD2 was observed in one case, while in two other cases partial loss (< 50%) of either CD3 or CD2 was observed. In 23 of 25 SS cases (92%) more than 50% of the neoplastic CD4⁺ T cells in the skin expressed PD-1, in 21 cases even more than 75%, including the case with only minimal atypia (Table 1). In the other two cases, PD-1 was expressed by approximately 25% in one case and less than 10% in the other. In the patient with < 10% PD-1⁺ neoplastic T cells in the skin infiltrate, the lymph node biopsy showed a diffuse population of PD-1⁺CD3⁺CD4⁺ neoplastic T cells. Serial skin sections showed that CXCL13 and BCL6 generally stained 25–50% of the PD-1⁺ cells.

DISCUSSION

The histologic diagnosis of SS in skin biopsies is one of the most challenging issues in dermatopathology. Recent studies of our group showed almost consistent expression of PD-1 by the neoplastic T cells of SS.⁸ In the present study the expression of PD-1 and other markers was investigated in skin biopsies from 30 patients with various types of EID, and compared with 25 patients with SS from our recent study.⁸ The main goal of the present study was to determine whether the number and distribution of PD-1⁺ cells could be used as an adjunct in the differential diagnosis between EID and SS.

As described previously, PD-1 was strongly expressed by the large majority of neoplastic CD4⁺ T cells in 23 of 25 patients with SS (92%), both in the dermis and in

the epidermis.⁸ In skin biopsies of EID, percentages of PD-1⁺ T cells varied between < 5% to > 50% of the total number of infiltrating dermal CD3⁺ T cells. They were most numerous in skin biopsies of patients with atopic erythroderma. Percentages > 50%, used in our previous study as a cut-off point for positivity in CTCL, were observed in only 4 of 30 cases (13%), including 3 cases of atopic erythroderma and one case with paraneoplastic erythroderma. Although double staining was not performed, examination of serial sections suggested that PD-1 was predominantly expressed by CD8⁺ T cells. In particular, the large majority of scattered PD-1⁺ intraepidermal lymphocytes were CD8 positive. BCL6 was expressed by < 5% up to 20% (median, 10%) of the dermal T cells, while CXCL13⁺ cells were few or absent. Expression of PD-1, but not of other TFH-cell markers BCL6 and CXCL13, suggest that these PD-1⁺ cells are activated T cells, and not TFH cells.

Other differences between skin biopsies of SS and EID concerned the density and cellular composition of the dermal infiltrates, the extent and phenotype of epidermotropic T cells, and loss of pan-T-cell markers. Consistent with previous reports, skin biopsies of SS showed mainly perivascular infiltrates, which were generally more pronounced and more monotonous than observed in EID biopsies.^{11,13,14} These infiltrates were predominantly composed of small-to-large atypical CD4⁺ T cells with hyperconvoluted nuclei (Sézary cells) and variable numbers of blast cells. In three cases of atopic erythroderma the dermal infiltrates also showed a predominance of slightly atypical small/medium-sized pleomorphic T cells and scattered blast cells and therefore a diagnosis of suspected CTCL had initially been considered. In addition, in one of them PD-1 was also expressed by > 50% of the dermal CD3⁺ T cells. However, percentages of CD8⁺ T cells varied between 40% and 60% in these cases, while percentages of admixed dermal CD8⁺ T cells in SS rarely exceeded 25%. Pautrier microabscesses containing CD4⁺ neoplastic T cells were observed in 10 of 25 cases of SS. Colonization of the basal layers of the epidermis, which is a characteristic feature of classical early-stage MF, was observed in only two SS cases, and in one case of paraneoplastic erythroderma. However, in this latter case, the intraepidermal T cells expressed CD8 and not CD4 as in SS.

Loss of CD7 is a common feature in CTCL, but has also been reported in a variety of benign inflammatory dermatoses, probably as a result of chronic T-cell activation.^{15,16} Unlike loss of other T-cell antigens, such as CD2, CD3 and CD5, loss of CD7 is therefore not considered as a useful criterion to differentiate between CTCL and inflammatory skin diseases.^{15,16} In the present study, loss of CD7 by more than 50% of the infiltrating T cells was observed in 16 of 24 cases of SS (66%), and only in 3 of 30 cases of EID (10%). In 13 of 24 cases of SS (54%) CD7 was expressed by ≤ 20% of the CD3⁺ T cells, while the percentages of CD7⁺ T cells in the 3 cases of EID varied between 30% and 40%. Expression of CD7 by ≤ 20% of the T cells

therefore strongly supports a diagnosis of SS. Loss of other pan-T-cell markers (CD2, CD3, CD5) was not observed in EID, and only rarely in SS.

In conclusion, PD-1 expression by > 50% of skin-infiltrating T cells in a patient with erythroderma is highly suggestive of a diagnosis of SS, but can also be observed —although uncommonly— in skin biopsies from patients with EID. In contrast to SS, in which PD-1 is expressed by CD4⁺ neoplastic T cells, our results suggest that in EID PD-1 is expressed mainly by activated dermal and epidermal CD8⁺ T cells. Partial loss of CD7 expression was common in both SS and EID, but expression of CD7 by ≤ 20% of the infiltrating T cells was found only in SS. Our results suggest that expression of PD-1 by > 50% of CD4⁺ T cells and expression of CD7 by ≤ 20% of the infiltrating T cells may be considered as valuable adjuncts in the differentiation between SS and EID.

REFERENCE LIST

1. Fife BT, Pauken KE. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Ann N Y Acad Sci* 2011; 1217: 45-59.
2. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 2010; 236: 219-42.
3. Roncador G, Garcia Verdes-Montenegro JF, Tedoldi S et al. Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. *Haematologica* 2007; 92: 1059-66.
4. Rodriguez-Pinilla SM, Atienza L, Murillo C et al. Peripheral T-cell lymphoma with follicular T-cell markers. *Am J Surg Pathol* 2008; 32: 1787-99.
5. Rodriguez-Pinilla SM, Roncador G, Rodriguez-Peralto JL et al. Primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol* 2009; 33: 81-90.
6. Cetinozman F, Jansen PM, Willemze R. Expression of programmed death-1 in primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma, cutaneous pseudo-T-cell lymphoma, and other types of cutaneous T-cell lymphoma. *Am J Surg Pathol* 2012; 36: 109-16.
7. Ally MS, Prasad Hunasehally RY, Rodriguez-Justo M et al. Evaluation of follicular T-helper cells in primary cutaneous CD4⁺ small/medium pleomorphic T-cell lymphoma and dermatitis. *J Cutan Pathol* 2013; 40: 1006-13.
8. Cetinozman F, Jansen PM, Vermeer MH et al. Differential Expression of Programmed Death-1 (PD-1) in Sezary Syndrome and Mycosis Fungoides. *Arch Dermatol* 2012; 148: 1379-85.
9. Wada DA, Wilcox RA, Harrington SM et al. Programmed death 1 is expressed in cutaneous infiltrates of mycosis fungoides and Sezary syndrome. *Am J Hematol* 2011; 86: 325-7.

10. Kantekure K, Yang Y, Raghunath P et al. Expression patterns of the immunosuppressive proteins PD-1/CD279 and PD-L1/CD274 at different stages of cutaneous T-cell lymphoma/mycosis fungoides. *Am J Dermatopathol* 2012; 34: 126-8.
11. Ram-Wolff C, Martin-Garcia N, Bensussan A et al. Histopathologic diagnosis of lymphomatous versus inflammatory erythroderma: a morphologic and phenotypic study on 47 skin biopsies. *Am J Dermatopathol* 2010; 32: 755-63.
12. Willemze R, Jaffe ES, Burg G et al. WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005; 105: 3768-85.
13. Sentis HJ, Willemze R, Scheffer E. Histopathologic studies in Sezary syndrome and erythrodermic mycosis fungoides: a comparison with benign forms of erythroderma. *J Am Acad Dermatol* 1986; 15: 1217-26.
14. Diwan AH, Prieto VG, Herling M et al. Primary Sezary syndrome commonly shows low-grade cytologic atypia and an absence of epidermotropism. *Am J Clin Pathol* 2005; 123: 510-5.
15. Alaibac M, Pigozzi B, Belloni-Fortina A et al. CD7 expression in reactive and malignant human skin T-lymphocytes. *Anticancer Res* 2003; 23: 2707-10.
16. Florell SR, Cessna M, Lundell RB et al. Usefulness (or lack thereof) of immunophenotyping in atypical cutaneous T-cell infiltrates. *Am J Clin Pathol* 2006; 125: 727-36.

5

DIFFERENTIAL EXPRESSION OF TOX BY SKIN-INFILTRATING T CELLS IN SÉZARY SYNDROME AND ERYTHRODERMIC DERMATITIS

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ABSTRACT

Background: The histopathologic differentiation between Sézary syndrome (SS) and erythrodermic dermatitis may be extremely difficult. In this immunohistochemical study, it was investigated if thymocyte selection-associated high mobility group box protein (TOX) and C-MYC can be used as additional diagnostic markers to differentiate between SS and erythrodermic dermatitis.

Methods: Paraffin-embedded skin biopsies from 15 patients with SS and 17 patients with erythrodermic dermatitis were stained and scored for TOX or C-MYC expression.

Results: Strong nuclear staining for TOX in more than 50% of skin-infiltrating T cells was observed in 13 of 15 SS cases (87%), whereas erythrodermic dermatitis cases showed weak nuclear staining in 11–50% (median, 25%) of the T cells; strong nuclear staining for TOX as found in SS was never observed in erythrodermic dermatitis. No significant differences in C-MYC expression between SS and erythrodermic dermatitis were found. In most patients of both groups, percentages of C-MYC positive cells varied between less than 10–25% of skin-infiltrating T cells.

Conclusions: Our results suggest that strong expression of TOX in more than 50% of skin-infiltrating T cells in erythrodermic skin is a useful marker in the differentiation between SS and erythrodermic dermatitis, whereas staining for C-MYC does not contribute to differential diagnosis.

INTRODUCTION

Sézary syndrome (SS) is a rare and aggressive type of cutaneous T-cell lymphoma (CTCL), characterized by a pruritic erythroderma and the presence of clonal neoplastic T cells (Sézary cells) in skin and peripheral blood.¹

The microscopic differentiation between SS and erythrodermic dermatitis may be extremely difficult.^{2,3} The histopathologic findings of SS are similar to that of mycosis fungoides (MF), but more commonly the entity shows a monotonous band-like or perivascular infiltrate in the papillary dermis, that is mainly composed of lymphocytes with atypical or cerebriform nuclei. Epidermotropism is present and Pautrier microabscesses may be found. However, in up to one third of SS cases, microscopic sections may show non-specific features of chronic dermatitis.^{4,5}

In a recent study, blind evaluation of hematoxylin and eosin-stained sections from skin biopsies of 18 patients with a CTCL, including 14 SS patients and 29 patients with erythrodermic dermatitis, correct differentiation between CTCL and erythrodermic dermatitis was made in approximately 50% of the cases.³ These observations indicate that there is an urgent need for diagnostic biomarkers for SS.

In a recent study of our group both programmed death-1 (PD-1; CD279) and CD7 proved valuable immunophenotypic markers in the differentiation between SS and erythrodermic dermatitis.⁶ Expression of PD-1 by more than 50% of the skin-infiltrating T cells was found in 23 of 25 SS cases (92%) and only in 4 of 30 erythrodermic dermatitis cases (13%). In SS, PD-1 was expressed by the neoplastic CD4⁺ T cells, whereas in erythrodermic dermatitis PD-1 was predominantly expressed by dermal and epidermal CD8⁺ T cells. Loss of CD7 by more than 50% of the skin-infiltrating T cells was found in 16 of 24 SS cases (66%) but also in 4 of 30 erythrodermic dermatitis cases (13%). However, expression of CD7 by 20% or less of infiltrating T cells was found only in SS (13 of 24 cases).

In this study, we used the same cohort of erythrodermic patients to investigate the differential diagnostic value of two other potentially useful biomarkers, namely thymocyte selection-associated high mobility group box protein (TOX) and C-MYC.

TOX belongs to a large family of chromatin-associated proteins. In T-cell development, TOX is highly expressed in the thymus during the transition of CD4⁺CD8⁺ precursors to CD4⁺ T cells but is normally not expressed by mature CD4⁺ T cells once they leave the thymus.^{7,8}

Recent studies have reported increased TOX expression by malignant CD4⁺ T cells in MF and SS skin biopsies, but not in benign inflammatory dermatoses.⁹⁻¹² In addition, Huang *et al.* showed that TOX mRNA expression is significantly enhanced in primary CD4⁺CD7⁻ cells from peripheral blood of SS patients, as compared to those cells from patients with benign inflammatory dermatosis.¹³ The increase of TOX mRNA expression was correlated with increased risk of disease progression and disease-specific mortality. It was suggested that TOX might contribute to the

development of CTCL.¹³ However, its biological effects on CTCL pathogenesis have not been explored. Studies focusing on TOX expression in skin of patients with extensive erythrodermic dermatitis have not been performed.

The rationale to consider C-MYC as a potential diagnostic marker results from previous studies showing a gain of 8q24 harboring the MYC gene in 41–75% of SS patients.^{14,15} Immunohistochemical studies for C-MYC expression in MF or SS are limited. Kanavaros *et al.* reported expression of C-MYC by 5–25% of the skin-infiltrating lymphoid cells in cases of early stage MF and SS, whereas higher percentages (25–50%) were found in one-third of patients with advanced MF.¹⁶ In a recent study, 12 of 13 (92%) patients with erythrodermic MF/SS showed positive staining for C-MYC in at most 15% of the dermal lymphocytic infiltrate cells.¹⁷

In this study, we therefore investigated if TOX and C-MYC can be used as additional markers to differentiate between SS and erythrodermic dermatitis.

METHODS

Patients

Paraffin-embedded skin biopsies from 15 patients with SS and 17 patients with erythrodermic dermatitis were selected for this study. The diagnosis of SS was based on the recent criteria of the World Health Organization – European Organization for Research and Treatment of Cancer classification.¹ The diagnosis of erythrodermic dermatitis in each patient was based on clinical and histopathological criteria, supplemented by immunophenotyping and clonality analysis of peripheral blood to exclude peripheral blood involvement by CTCL. The erythrodermic dermatitis group included four patients with atopic erythroderma, three patients with erythrodermic psoriasis, nine patients with idiopathic erythroderma and one patient with paraneoplastic erythroderma. Review of clinical records revealed that none of the patients developed a lymphoma after a median follow-up of 46 months (range, 7–332 months). The study complied with the Declaration of Helsinki and was performed in accordance with the Dutch Code and Leiden University Medical Center guidelines on leftover material.

Histopathology and immunohistochemistry

Sections from all skin biopsies had routinely been stained with hematoxylin-eosin and with monoclonal antibodies against T cell-associated antigens (CD2, CD3, CD4, CD5, CD7 and CD8), B cell-associated antigens (CD20 and/or CD79a) and CD68 and CD1a to differentiate between CD4⁺ T cells and CD4⁺ histiocytes and Langerhans cells/dendritic cells, respectively. For the purpose of this study, sections from all patients were stained for TOX and C-MYC.

Immunohistochemical staining was performed on 4- μ m sections using standard procedures. After antigen retrieval by heating for 10–12 minutes in 1.0 mmol/L ethylenediaminetetraacetic acid (pH 8.0) or 10 mmol/L citrate buffer (pH 6.0), tissue sections were incubated overnight with antibodies against TOX (Sigma-Aldrich, Zwijndrecht, The Netherlands) and C-MYC (Abcam, Cambridge, United Kingdom). Sections were then incubated for 30 min with BrightVision Poly-horseradish peroxidase, and subsequently incubated with diaminobenzidine (DAB) solution (Sigma-Aldrich) for 10 minutes. Finally, all slides were counterstained with Mayer haematoxylin.

The percentages of T cells expressing TOX or C-MYC were scored, as less than 10%, 11–25%, 26–50%, 51–75% and more than 75%. These percentages had been estimated independently by three observers (FC, PMJ, RW). In the few cases in which there were disagreement sections, they were viewed jointly by all authors and consensus was reached.

RESULTS

Sézary syndrome

The results of the immunohistochemical stainings are summarized in Table 1. This table also contains the results of PD-1 staining and the presence or absence of antigen loss from our previous study.⁶

Skin biopsies from patients with SS characteristically showed perivascular to band-like infiltrates in the papillary dermis. More diffuse infiltrates extending into the reticular dermis were observed in four cases. Epidermotropic neoplastic CD4⁺ T cells with (n = 5) or without (n = 5) Pautrier microabscesses were found in ten cases. The dermal infiltrates were predominantly composed of small to large atypical CD4⁺ T cells with hyperconvoluted nuclei (Sézary cells) and variable numbers of blast cells. In 2 of 15 cases (nos. 6 and 15) the cellular atypia was minimal, and a diagnosis of CTCL was at most suspected. Percentages of admixed CD8⁺ T cells varied between less than 5% and almost 50%, but a percentage more than 30% was observed in only two cases (nos. 6 and 15).

Strong nuclear staining for TOX was observed in more than 50% of the skin-infiltrating T cells in 13 of 15 (87%) with percentages more than 75% in ten of them (Table 1). Intraepidermal neoplastic T cells consistently showed strong nuclear staining for TOX (Figure 1). In two cases (nos. 6 and 15), weak nuclear staining in 25–30% of the skin-infiltrating T cells was found (Figure 2). C-MYC was expressed by less than 10% of the skin-infiltrating T cells in 5 of 15 cases, by 11–25% in eight cases and in 26–50% in two cases. Strong nuclear C-MYC staining was particularly observed in large Sézary cells and blast cells (Figure 1). In addition, epidermal basal cells showed strong nuclear C-MYC staining, serving as a useful internal control.

Table 1. Summary of the immunohistochemical stainings

Case	Diagnosis	TOX (%)	C-MYC (%)	PD-1 (%)	Antigen loss
1	SS	++++	+	++++	–
2	SS	++++	+	++++	CD7, CD2
3	SS	++++	+	++++	CD7
4	SS	+++	–	++++	CD7
5	SS	++++	+	++++	CD7
6	SS *	++ (w)	–	+++	–
7	SS	++++	++	++++	CD7
8	SS	+++	–	+++	–
9	SS	++++	++	++++	CD7
10	SS	++++	+	++++	CD7
11	SS	++++	+	+	–
12	SS	++++	+	+++	CD7, CD2
13	SS	+++	–	++++	CD7
14	SS	++++	+	++++	CD7
15	SS *	++ (w)	–	+++	CD7
16	AE †	+	–	+	–
17	AE	+	–	++	–
18	AE †	+	–	+++ ‡	–
19	AE †	++ (w)	–	+++ ‡	CD7
20	PSOR	+	+	++	–
21	PSOR	+	–	+	–
22	PSOR	+	–	–	–
23	IE	+	+	–	–
24	IE	++ (w)	–	–	–
25	IE	++ (w)	+	–	–
26	IE	+	+	–	–
27	IE	++ (w)	–	–	–
28	IE	+	+	+	–
29	IE	+	+	+	CD7
30	IE	+	+	+	–
31	IE	+	–	+	–
32	PARA	+	+	+	–

AE: atopic erythroderma; IE: idiopathic erythroderma; PARA: paraneoplastic erythroderma; PD-1, programmed death-1; PSOR: psoriatic erythroderma; SS: Sézary syndrome.

TOX, C-MYC and PD-1: –, <10%; +, 11–25%; ++, 26–50%; +++, 51–75%; +++++, >75%

Antigen loss: loss of CD2, CD3, CD4, CD5 or CD7 expression by more than 50% of the (neoplastic) T cells; –: no loss.

* SS cases showing no or minimal cellular atypia

† Cases showing considerable numbers of small to medium-sized atypical T cells

‡ CD8⁺ T cells; (w): weak staining

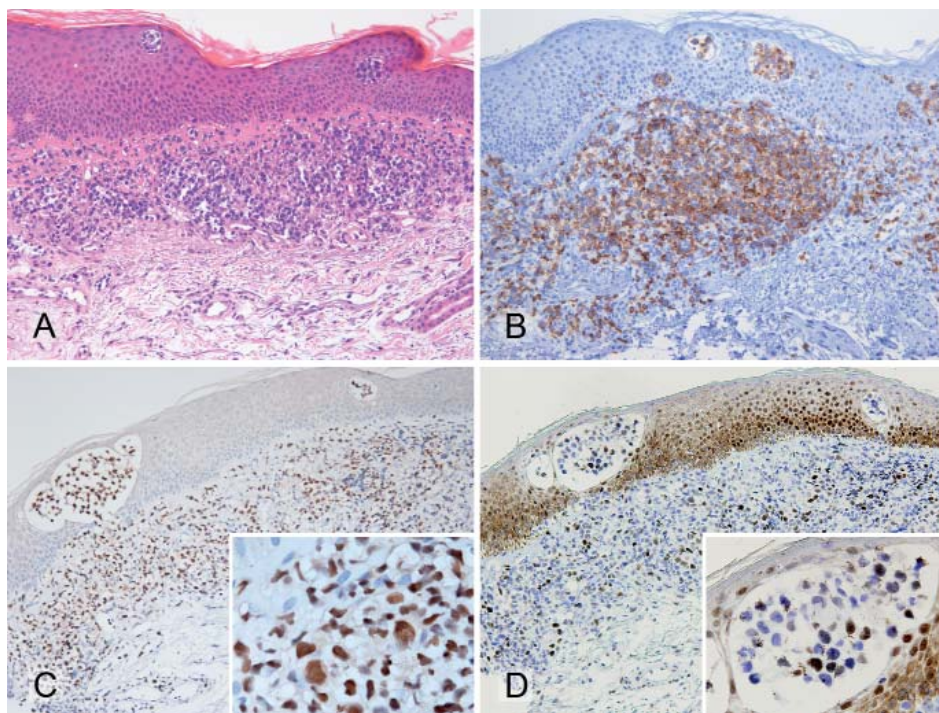


Figure 1. Histopathologic features of a representative Sézary syndrome patient with strong TOX expression. A) The haematoxylin-eosin staining shows an infiltrate in the superficial dermis with epidermal Pautrier microabscesses. B) Expression of CD3. C) More than 50% of the skin-infiltrating T cells show strong nuclear staining for TOX, particularly in large Sézary cells and blast cells. D) Less than 25% of the skin-infiltrating T cells expressed C-MYC. The insets in (C) and (D) show higher magnifications of corresponding areas. (A – D: $\times 100$, inset in C: $\times 630$, inset in D: $\times 400$).

Erythrodermic dermatitis

Biopsy specimens of erythrodermic dermatitis generally showed a sparse to moderately dense perivascular to band-like infiltrate in the superficial dermis, and the infiltrate was generally much less pronounced than observed in SS. Intraepidermal T cells were few or absent, and in some cases could only be recognized in immunostained sections. The superficial dermal infiltrate was mainly composed of small lymphocytes admixed with variable numbers of histiocytes, and in cases of atopic and idiopathic erythroderma with eosinophils. In three cases of atopic erythroderma (nos. 16, 18 and 19), the dermal infiltrate showed a considerable number of slightly atypical small to medium-sized pleomorphic T cells and scattered blast cells, and a diagnosis of suspected CTCL had initially been made. Percentages of CD8⁺ T cells in the dermal infiltrates varied between 15% and 75% (median, 30%) of the dermal CD3⁺ T cells.

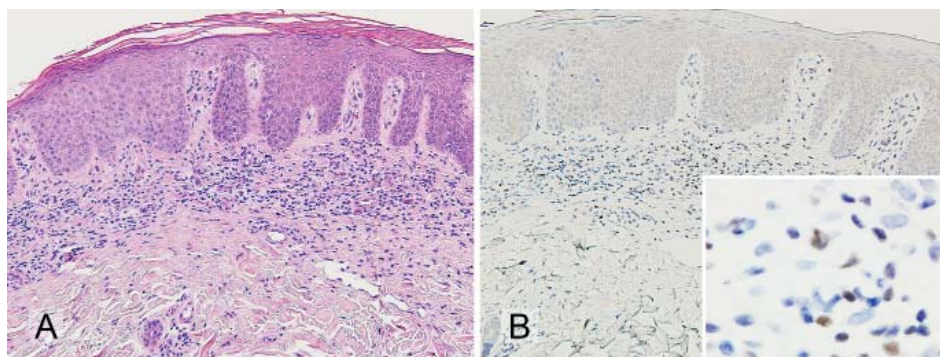


Figure 2. Histopathologic features of a Sézary syndrome patient with weak TOX expression. A) The haematoxylin-eosin staining shows a perivascular to band-like infiltrate in the papillary dermis. B) Weak expression of TOX by 25–30% of the skin-infiltrating T cells. (A and B: $\times 100$ and inset in B: $\times 630$).

Weak nuclear staining for TOX was observed in 11–25% of the infiltrating T cells in 13 of 17 cases, and in 26–50% in four cases (Figure 3). Strong nuclear staining for TOX as found in SS was never observed in erythrodermic dermatitis.

Nuclear staining for C-MYC was detected in 11–25% of both small and scattered larger T cells in 8 of 17 cases and by less than 10% in nine cases. In three cases, they were completely lacking (nos. 17, 18 and 19), whereas the epidermal basal cells still showed strong nuclear staining.

DISCUSSION

In this study, strong nuclear staining for TOX by more than 50% of the skin-infiltrating T cells was found in 13 of 15 Sézary cases (87%), whereas all erythrodermic dermatitis cases showed weak nuclear staining of TOX varying between 11% and 50% of the T cells. The two remaining SS cases (nos. 6 and 15) showed weak nuclear TOX expression at a similar level as seen in erythrodermic dermatitis patients. Interestingly, both cases showed minimal atypia and had high numbers of admixed CD8⁺ T cells. It should however be noted that in our previous study both cases had shown expression of PD-1 by more than 50% of the skin-infiltrating T cells. Alternatively, in one SS patient with expression of PD-1 in only 10% of neoplastic T cells (no. 11), more than 75% of the neoplastic T cells showed strong nuclear TOX staining, suggesting that combining both markers may be diagnostically helpful.

In a previous study, we observed that — in contrast to SS — PD-1 is uncommonly expressed by the neoplastic T cells in skin biopsies from erythrodermic MF. Expression of PD-1 by more than 50% of the neoplastic T cells was found in only one of eight cases.¹⁸ Examination of skin biopsies from seven of these cases showed

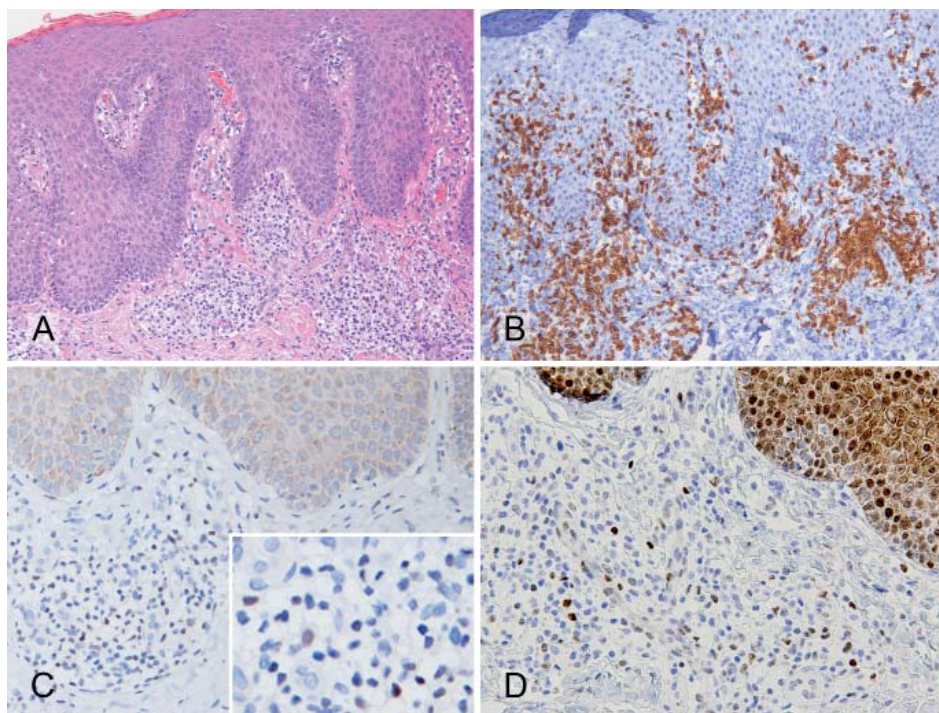


Figure 3. Histopathologic features of a representative patient with erythrodermic psoriasis. A) The haematoxylin-eosin staining of the lesion shows acanthosis and a dermal lymphocytic infiltrate. B) Expression of CD3. C) Weak nuclear staining for TOX in a small proportion of the infiltrating T cells. D) Nuclear staining for C-MYC in scattered infiltrating T cells. (A and B: $\times 100$, C and D: $\times 200$ and inset in C: $\times 630$).

strong nuclear staining for TOX by more than 50% of the malignant T cells in six of them (Willemze *et al.*; unpublished observations 2015). These results are similar to those of this study in SS and indicate that TOX is a useful biomarker to differentiate erythrodermic dermatitis from both SS and erythrodermic MF.

Our results are consistent with recent studies showing strong nuclear staining for TOX by atypical $CD4^+$ T cells, both in dermis and epidermis, in SS and MF skin biopsies compared with benign inflammatory dermatoses and normal skin.^{9,10,12} In addition, previous studies have shown increased mRNA expression levels for TOX in both SS and MF as well.⁹⁻¹³

The high expression of TOX in CTCL is as yet unexplained. In normal T-cell development mature $CD4^+$ T cells do not express TOX after leaving the thymus. Whether the TOX expression found in SS and other types of CTCL may be because of an impaired regulated maturation status or to re-expression during the formation into memory T cell has yet to be determined.⁷

No significant differences in C-MYC expression between SS and erythrodermic dermatitis were found. In both groups about half of the cases showed C-MYC positivity in 11–25% of the skin-infiltrating T cells. Higher percentages (25–50%) were found in only two cases of SS. Similarly, Kavanos *et al.* reported percentages of 11–25% in three of three (100%) SS cases.¹⁶

Previous studies reported a gain of 8q24 harboring the MYC gene in 41–75% of SS patients.^{14,15} In a recent European multicenter study on 59 Sézary patients gain for MYC was found in 23 of 58 (40%) SS cases as well (Boonk *et al.* 2015, submitted manuscript). However, using the quantitative polymerase chain reaction technique, no differences in MYC gene expression levels were found between Sézary cells and CD4⁺ T cells from erythrodermic dermatitis patients. Three of the 15 SS cases (nos. 2, 10 and 11) from this study had been included in the previous study and showed a gain for MYC. However, all of them showed C-MYC staining in only 11–25% of the skin-infiltrating T cells, similar to the other SS cases. Why the gain for MYC does not lead to increased gene and protein expression is as yet unexplained.

In conclusion, strong expression of TOX, but not C-MYC, can be another useful adjunct in the differentiation between SS and erythrodermic dermatitis.

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REFERENCE LIST

1. Willemze R, Jaffe ES, Burg G *et al.* WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005; 105(10):3768-3785.
2. Diwan AH, Prieto VG, Herling M, Duvic M, Jone D. Primary Sezary syndrome commonly shows low-grade cytologic atypia and an absence of epidermotropism. *Am J Clin Pathol* 2005; 123(4):510-515.
3. Ram-Wolff C, Martin-Garcia N, Bensussan A, Bagot M, Ortonne N. Histopathologic diagnosis of lymphomatous versus inflammatory erythroderma: a morphologic and phenotypic study on 47 skin biopsies. *Am J Dermatopathol* 2010; 32(8):755-763.
4. Sentis HJ, Willemze R, Scheffer E. Histopathologic studies in Sezary syndrome and erythrodermic mycosis fungoides: a comparison with benign forms of erythroderma. *J Am Acad Dermatol* 1986; 15(6):1217-1226.

5. Trotter MJ, Whittaker SJ, Orchard GE, Smith NP. Cutaneous histopathology of Sezary syndrome: a study of 41 cases with a proven circulating T-cell clone. *J Cutan Pathol* 1997; 24(5):286-291.
6. Cetinozman F, Jansen PM, Willemze R. Expression of programmed death-1 in skin biopsies of benign inflammatory vs. lymphomatous erythroderma. *Br J Dermatol* 2014; 171(3):499-504.
7. Wilkinson B, Chen JY, Han P, Rufner KM, Goularte OD, Kaye J. TOX: an HMG box protein implicated in the regulation of thymocyte selection. *Nat Immunol* 2002; 3(3):272-280.
8. Aliahmad P, Seksenyan A, Kaye J. The many roles of TOX in the immune system. *Curr Opin Immunol* 2012; 24(2):173-177.
9. Zhang Y, Wang Y, Yu R et al. Molecular markers of early-stage mycosis fungoides. *J Invest Dermatol* 2012; 132(6):1698-1706.
10. McGirt LY, Adams CM, Baerenwald DA, Zwerner JP, Zic JA, Eischen CM. miR-223 regulates cell growth and targets proto-oncogenes in mycosis fungoides/cutaneous T-cell lymphoma. *J Invest Dermatol* 2014; 134(4):1101-1107.
11. Huang Y, Litvinov IV, Wang Y et al. Thymocyte selection-associated high mobility group box gene (TOX) is aberrantly over-expressed in mycosis fungoides and correlates with poor prognosis. *Oncotarget* 2014; 5(12):4418-4425.
12. Morimura S, Sugaya M, Suga H et al. TOX expression in different subtypes of cutaneous lymphoma. *Arch Dermatol Res* 2014; 306(9):843-849.
13. Huang Y, Su MW, Jiang X, Zhou Y. Evidence of an oncogenic role of aberrant TOX activation in cutaneous T cell lymphoma. *Blood* 2014.
14. Vermeer MH, van Doorn R, Dijkman R et al. Novel and highly recurrent chromosomal alterations in Sezary syndrome. *Cancer Res* 2008; 68(8):2689-2698.
15. Laharanne E, Oumouhou N, Bonnet F et al. Genome-wide analysis of cutaneous T-cell lymphomas identifies three clinically relevant classes. *J Invest Dermatol* 2010; 130(6):1707-1718.
16. Kanavaros P, Ioannidou D, Tzardi M et al. Mycosis fungoides: expression of C-myc p62 p53, bcl-2 and PCNA proteins and absence of association with Epstein-Barr virus. *Pathol Res Pract* 1994; 190(8):767-774.
17. Goswami M, Duvic M, Dougherty A, Ni X. Increased Twist expression in advanced stage of mycosis fungoides and Sezary syndrome. *J Cutan Pathol* 2012; 39(5):500-507.
18. Cetinozman F, Jansen PM, Vermeer MH, Willemze R. Differential expression of programmed death-1 (PD-1) in Sezary syndrome and mycosis fungoides. *Arch Dermatol* 2012; 148(12):1379-1385.

6

PROGRAMMED DEATH-1 EXPRESSION IN CUTANEOUS B-CELL LYMPHOMA

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ABSTRACT

Background: Numbers of programmed death-1 (PD-1) positive T cells have prognostic significance in some types of nodal B-cell lymphomas, but data on PD-1 expression in cutaneous B-cell lymphoma (CBCL) are few. In this study we determined the expression and distribution of PD-1 on neoplastic B cells and reactive T cells in skin sections from primary CBCLs.

Methods: By means of immunohistochemical staining, PD-1 expression was investigated in skin biopsies from 10 patients with primary cutaneous marginal zone lymphoma (PCMZL), 18 patients with primary cutaneous follicle center lymphoma (PCFCL) and 12 patients with primary cutaneous diffuse large B-cell lymphoma–leg type (PCLBCL–LT).

Results: Neoplastic B cells were negative for PD-1 in all cases, except for two cases of PCLBCL–LT. The frequency of PD-1⁺ T cells was significantly higher in PCFCL than in PCMZL and PCLBCL–LT, accounting for 20%, 10% and 3% of the total number of infiltrating cells, and 60%, 20% and 15% of the total number of CD3⁺ T cells, respectively.

Conclusions: PD-1 is rarely expressed by the neoplastic B cells in CBCL. High percentages of PD-1⁺ T cells, particularly if found outside germinal centers, support a diagnosis of PCFCL.

INTRODUCTION

Programmed death-1 (PD-1; CD279) belongs to the CD28/CTLA-4 receptor family and is expressed by CD4⁺ and CD8⁺ T cells after activation. Binding of PD-1 to its ligands PD-L1 (CD274) or PD-L2 (CD273) inhibits T-cell functions, such as proliferation, cytokine production and cytotoxic activity.^{1,2} Follicular helper T (TFH) cells are a special subset of CD4⁺ T cells, which have a constitutive high expression of PD-1. TFH are important for the formation of germinal centers and regulate the differentiation of germinal-center B cells into plasma cells and memory B cells.^{3,4} Interaction between PD-1 on the TFH cells and PD-L1 on the germinal-center B cells appears to enhance, rather than inhibit the germinal center reaction, however, the exact mechanism is not known.^{3,4}

Several studies investigated PD-1 expression in nodal B-cell non-Hodgkin lymphomas (B-NHLs). Except for small lymphocytic lymphoma and rare cases of nodal follicular B-cell lymphoma and diffuse large B-cell lymphoma, PD-1 is generally not present on neoplastic cells in B-NHLs.⁵⁻⁷ However, PD-1 is detectable on variable amounts of tumor infiltrating lymphocytes, in particular in follicular lymphoma.⁷⁻¹¹ Several studies reported an association in these nodal follicular lymphomas between increased numbers of PD-1⁺ T cells and a significantly improved survival, while in one study an association with a poorer outcome was found.⁷⁻¹⁰ Information on the expression of PD-1 in primary cutaneous B-cell lymphomas (CBCLs) is limited. Fanoni *et al.* reported positive immunohistochemical staining for PD-1 in primary cutaneous follicle center lymphoma (PCFCL), but not in primary cutaneous marginal zone lymphoma (PCMZL) and primary cutaneous diffuse large B-cell lymphoma–leg type (PCLBCL–LT).¹² However, the authors did not mention whether neoplastic B cells or reactive T cells were stained. In a very recent study, PD-1 was found to stain tumor-infiltrating T cells, but no neoplastic B cells in these three types of CBCL.¹³

In this study, we investigated the expression of PD-1 in skin sections from primary CBCLs. The aim of this study was to find out if PD-1 is expressed by the neoplastic B cells of these lymphomas. In addition, the number and distribution of PD-1⁺ T cells was investigated and correlated with clinical behavior.

METHODS

Patients

Paraffin-embedded skin biopsies from 10 patients with PCMZL, 18 patients with PCFCL and 12 patients with PCLBCL–LT were selected for this study. In addition, five cases of cutaneous lymphoid hyperplasia (pseudo-B-cell lymphoma) were included as controls. All skin samples were collected from the archives of the Department of Pathology, Leiden University Medical Center (LUMC) according to the “Dutch Code

Table 1. Clinical characteristics

	PCMZL	PCFCL	PCDLBCL-LT
Number of cases	10	18	12
Male / Female	6/4	12/6	6/6
Age at diagnosis (years)			
Median	48	57	78
Range	23–78	36–82	53–91
Localization			
Head	0	6	0
Trunk	9	11	1
Leg (s)	1	2	11
Arm	4	2	1
Multifocal	5	5	3
Follow-up (months)			
Median	24	76	39
Range	4–110	2–160	2–109
Current status			
A ⁰	4	10	4
A ⁺	5	6	0
D ⁰	1	2	3
D ⁺	0	0	5

PCMZL, primary cutaneous marginal zone lymphoma; PCFCL, primary cutaneous follicle center lymphoma; PCLBCL-LT, primary cutaneous diffuse large B-cell lymphoma-leg type; A⁺, indicates alive with disease; A⁰, alive without disease; D⁺, died of lymphoma; D⁰, died of unrelated cause.

for Proper Secondary Use of Human Tissue”, which is approved by the Medical Ethics Committee of the LUMC. All CBCL cases had been reviewed by an expert panel of dermatologists and pathologists during one of the quarterly meetings of the Dutch Cutaneous Lymphoma group, and were classified according to the criteria of the World Health Organization – European Organization of Research and Treatment of Cancer (WHO–EORTC) classification for primary cutaneous lymphomas.¹⁴ All patients were adequately staged; physical examination, blood count, computed tomography scan and bone marrow biopsy were performed and showed no signs of extracutaneous disease manifestation at the time of diagnosis. The main clinical characteristics of these three groups of CBCL are presented in Table 1.

Histology and Immunohistochemistry

Sections from all biopsies had routinely been stained with hematoxylin-eosin and immunostainings to detect B-cell-associated antigens (CD20, CD79a, cytoplasmic immunoglobulins), germinal center B-cell-associated antigens (BCL6 and CD10), T-cell-associated antigens (CD3, CD4, and CD8), BCL2 and IRF4/MUM-1.¹⁵ For the purpose of this study sections from all patients were stained for PD-1 and CXCL13. Staining procedures for PD-1 and CXCL13, using antibodies from R&D Systems

(Abingdon, UK), have previously been described.^{16,17} The percentages of CD3⁺ T-cells, PD-1⁺ cells and CXCL13⁺ cells were expressed as a percentage of the total number of skin-infiltrating cells (both reactive and neoplastic). Using serial sections percentages of these cells were estimated independently by three observers (FC, PJ, RW) to the nearest 5% for the whole section. In the few cases in which there was disagreement, sections were read jointly and consensus was reached.

Statistical analysis

Statistical significance was determined with one-way analysis of variance (ANOVA) and Student's t-test using SPSS software, with $p < 0.05$ considered as significant.

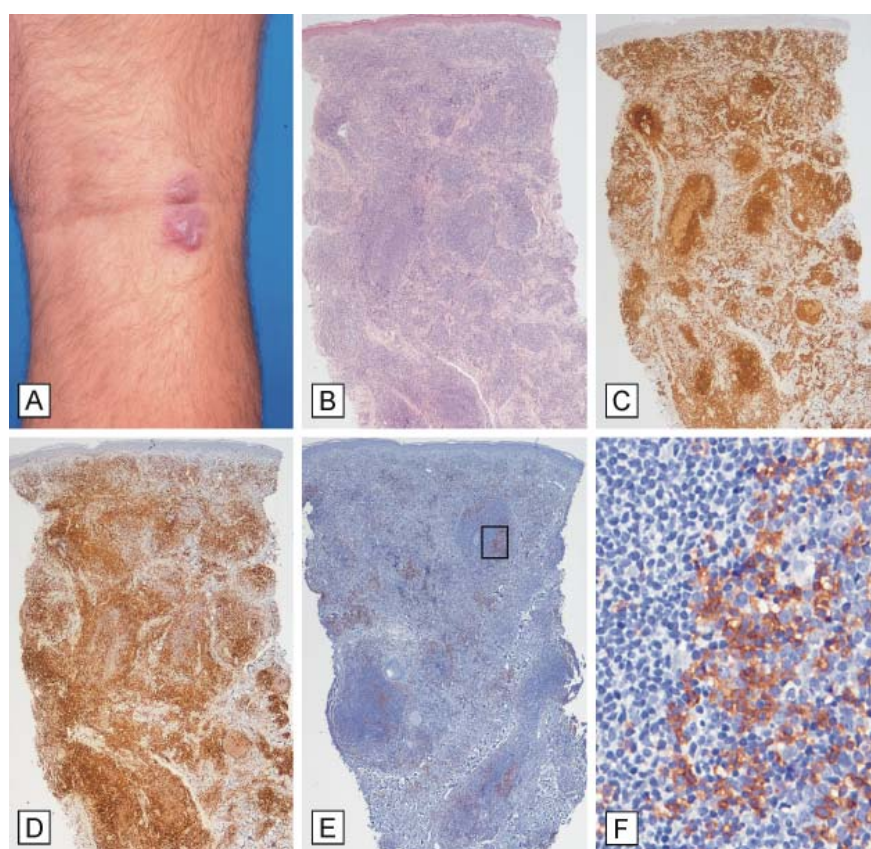


Figure 1. Histopathologic features of a representative primary cutaneous marginal zone lymphoma (PCMZL) patient. (A) Patient presenting with a tumor in the right popliteal area. (B) Hematoxylin-eosin staining of the lesion showing nodular to diffuse infiltrates containing (C) CD79a⁺ neoplastic B cells and reactive follicles, and (D) high numbers of admixed T cells. (E) A small proportion of the T cells showed programmed death-1 (PD-1) positivity; these cells were typically found within reactive germinal centers. (F) A higher magnification of the marked area in (E) showing a germinal center containing PD-1⁺ T cells. (B–E, original magnification x25; F, original magnification x100).

RESULTS

PD-1 expression in PCMZL

PCMZL showed nodular to diffuse infiltrates containing variable numbers of marginal zone B cells, lymphoplasmacytoid cells and plasma cells and considerable numbers of admixed T cells (Figure 1). Reactive follicles were observed in 8 of 10 cases. Plasma cells and/or lymphoplasmacytoid cells were characteristically found at the periphery of the infiltrates or at the subepidermal border showing either kappa or lambda monotypic immunoglobulin light chain expression. The neoplastic B cells, including marginal zone B cells and plasma cells, were PD-1 negative in all cases (Table 2).

Admixed CD3⁺ T cells were numerous, in all cases comprising 50–75% (median, 65%) of the total infiltrate (Figure 1). The proportion of PD-1⁺ T cells varied between 10% and 20% (median, 10%; mean, 13%) of the total infiltrate, and made up 15–30% (median, 20%) of the CD3⁺ T cells (Table 2). Examination of serial sections showed that CXCL13 generally stained 25–50% of the PD-1⁺ cells. These PD-1⁺ T cells were particularly found within reactive germinal centers and less frequently outside, and were spatially not associated with plasma cells (Figure 1). A very similar pattern was found in five cases of cutaneous pseudo-B-cell lymphoma. In these reactive lymphoid follicles PD-1⁺ T cells showed the same distribution as in normal reactive lymph nodes or tonsils, routinely included on the same glass slide as positive control. Clusters of four to six PD-1⁺ T cells rosetting around large B cells were regularly observed.

Table 2. Frequency of admixed CD3⁺ T cells and PD-1⁺ T cells

Type of lymphoma	Number	Reactive T lymphocytes		
		CD3 ⁺ *	PD-1 ⁺ †	PD-1 ⁺ /CD3 ⁺ ‡
		median (range)	median (range)	median (range)
PCMZL	10	65% (50–75%)	10% (10–20%)	20% (15–30%)
PCFCL	18	35% (10–50%)	20% (5–40%)	60% (25–90%)
> follicular / follicular and diffuse	9	40% (15–50%)	25% (15–40%)	65% (30–90%)
> diffuse	9	25% (10–50%)	10% (5–30%)	50% (25–75%)
PCLBCL–LT	12	13% (5–25%)	3% (1–15%)	15% (5–50%)

PCMZL, primary cutaneous marginal zone lymphoma; PCFCL, primary cutaneous follicle center lymphoma; PCLBCL–LT, primary cutaneous diffuse large B-cell lymphoma–leg type; PD-1, programmed death-1.

*proportion of CD3⁺ reactive T cells expressed as percentage of total number of infiltrating cells.

†proportion of PD-1⁺ reactive T cells expressed as percentage of total number of infiltrating cells.

‡proportion of PD-1⁺ cells expressed as percentage of reactive CD3⁺ T lymphocytes.

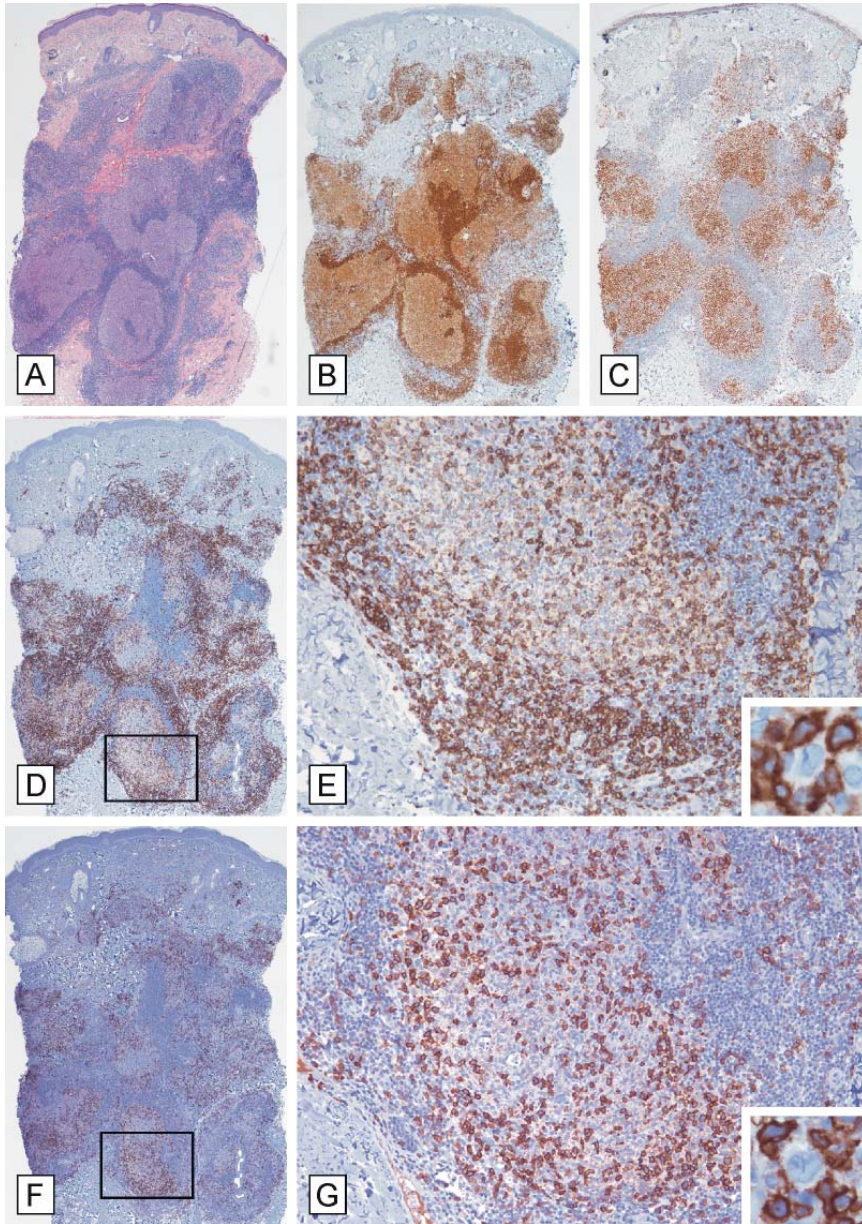


Figure 2. Representative histopathologic features of a primary cutaneous follicle center lymphoma (PCFCL) patient with a mainly follicular growth pattern. (A) Hematoxylin-eosin staining of the lesion showing nodular infiltrates containing neoplastic follicles with irregular mantles. (B) Neoplastic cells are CD79a positive. (C) BCL6 positive neoplastic germinal centers. (D and E) The neoplastic B cells are admixed with considerable numbers of small CD3⁺ T cells, of which (F and G) many were programmed death-1 (PD-1) positive. The insets in (E) and (G) are showing rosettes of CD3⁺ T cells and PD-1⁺ cells around large B cells, respectively. (A–D and F, original magnification x25; E and G, original magnification x100; inserts, original magnification x400).

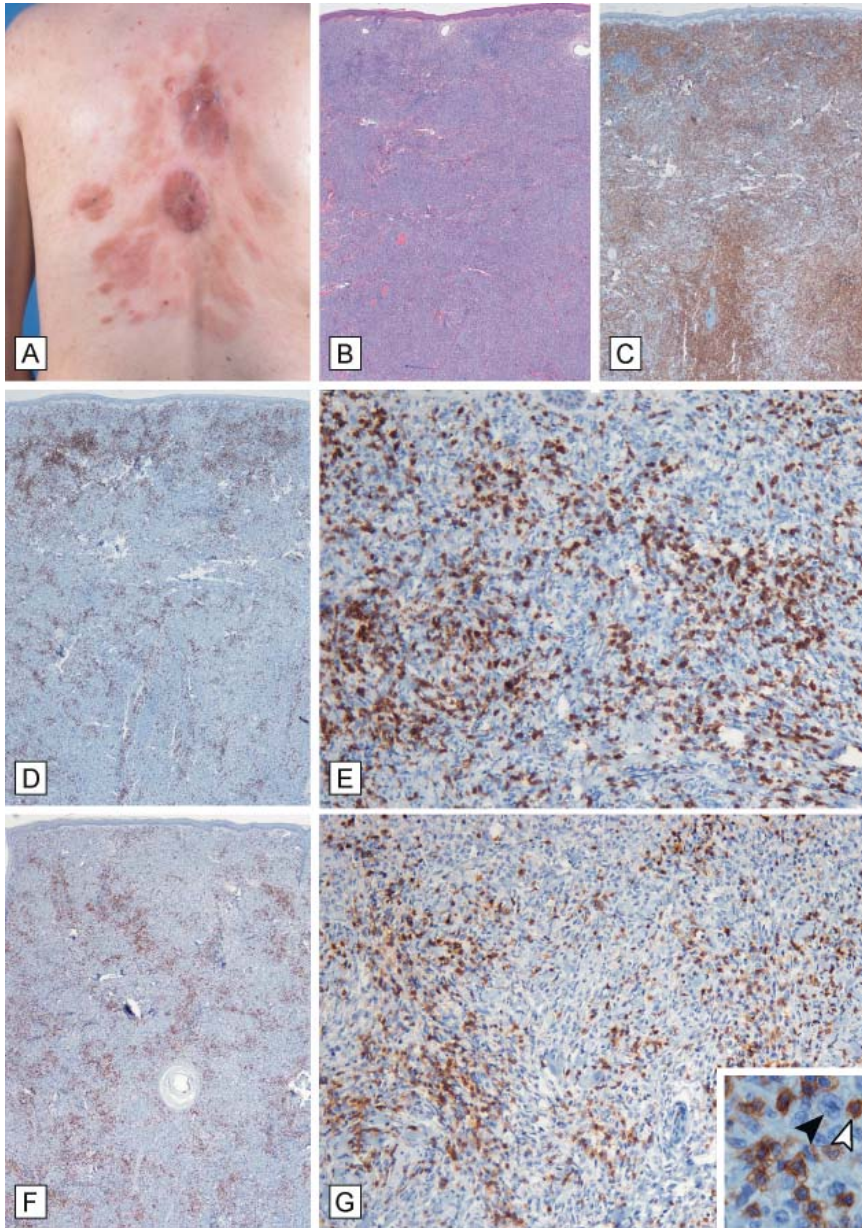


Figure 3. Representative histopathologic features of a primary cutaneous follicle center lymphoma (PCFCL) patient with a diffuse growth pattern. (A) Patient presenting with confluent tumors on the back. (B) Hematoxylin-eosin staining of the lesion showing diffuse infiltrates containing (C) CD79a⁺ neoplastic B cells. (D and E) Neoplastic B cells are admixed with considerable numbers of small CD3⁺ T cells, of which (F and G) many were programmed death-1 (PD-1) positive. The inset in (G) is showing the absence of PD-1 on neoplastic B cells (black arrowhead) and strong PD-1 expression on T cells (white arrowhead). (B–D and F, original magnification x25; E and G, original magnification x100; insert, original magnification x400).

PD-1 expression in PCFCL

PCFCL showed nodular to diffuse infiltrates containing medium-sized to large CD20⁺/CD79a⁺ cleaved cells (large centrocytes), variable numbers of centroblasts and considerable numbers of admixed small T cells (Figures 2 and 3). On the basis of routine histology and immunostains recognizing (remnants of) follicular dendritic cell networks (CD35 and CD21) these PCFCL showed either a purely follicular (n = 1), a follicular and diffuse (n = 8) or a diffuse growth pattern (n = 9). Characteristically, the neoplastic B cells expressed BCL6 (18/18 cases), but were negative for BCL2 (17/18 cases) and MUM-1 (18/18 cases), while CD10 was (partly) expressed in 6 of 16 cases investigated. Neoplastic B cells were PD-1 negative in all 18 cases (Table 2).

The proportion of admixed CD3⁺ T cells varied between 10% and 50% (median, 35%) of the total infiltrate, and was significantly lower than in PCMZL ($p < 0.01$). PD-1⁺ cells made up 5–40% of the total infiltrate (median, 20%) and were more frequent in cases with a (partly) follicular growth pattern than in cases with a diffuse growth pattern (Table 2). CXCL13 stained 25–50% of the PD-1⁺ cells. Examination of serial sections showed that PD-1 was expressed by more than 50% of the CD3⁺ T cells in 14 of 18 cases (Figures 2 and 3). The median percentage of 65% (range, 25–90%) was significantly higher than observed in PCMZL ($p < 0.01$) (Table 2). These PD-1⁺ T cells were particularly located within germinal centers or in areas with remnants of follicular dendritic cell networks. As in PCMZL, clusters of PD-1⁺ T cells rosetting around large B cells were regularly observed (Figure 2).

PD-1 expression in PCLBCL–LT

PCLBCL–LT biopsies showed diffuse infiltrates of centroblasts and immunoblasts, characteristically expressing BCL2 (11/11 cases) and MUM1 (10/12 cases), while BCL6 was expressed by 8 of 12 cases. Staining for CD10 was consistently negative. In 2 of 12 cases (17%) the neoplastic B cells showed weak PD-1 positivity (30% and 60% of the tumor cells, respectively; Table 1; Figure 4).

There were few reactive CD3⁺ T cells (median, 13%; range, less than 5–25%) and significantly less than in PCMZL ($p < 0.01$) and PCFCL ($p = 0.01$; Table 2; Figure 5). In 10 of 12 cases scattered PD-1⁺ cells made up less than 5% of the total infiltrate, while CXCL13⁺ cells were observed only occasionally. Serial sections showed that PD-1 was expressed by 5% to 25% of CD3⁺ T cells in 11 of 12 cases. In one patient, a 53-year-old man presenting with tumor on the trunk, CD3⁺ T cells made up 25% of the total infiltrate and 50% of these CD3⁺ T cells stained for PD-1. Compared to the nine PCFCL with a diffuse growth pattern, which also showed a diffuse population of large neoplastic B cells, these twelve PCLBCL–LT showed significantly less admixed T cells expressing PD-1 (50% versus 15%, respectively; $p = 0.01$).

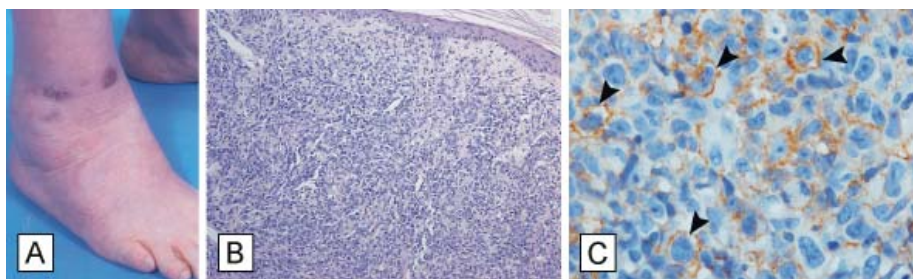


Figure 4. Histopathologic features of a primary cutaneous diffuse large B-cell lymphoma–leg type (PCLBCL–LT) patient with programmed death-1 (PD-1)⁺ neoplastic B cells. (A) Patient presenting with tumors on the dorsum of the right feet. (B) Hematoxylin-eosin staining of the lesion showing diffuse infiltrates. (C) A considerable number of the neoplastic B cells in the lesion showed weak PD-1 positivity (some positive cells are indicated by black arrowheads). (B, original magnification x25; C, original magnification x200).

DISCUSSION

In this study we investigated PD-1 expression in three types of primary CBCL: PCMZL, PCFCL and PCLBCL–LT. PD-1 was not expressed by the neoplastic B cells in these CBCL, except for two cases of PCLBCL–LT showing weak PD-1 staining in 30% and 60% of the neoplastic B cells, respectively. This observation is consistent with studies in nodal B-cell NHL, which showed that PD-1 is not or rarely expressed by the neoplastic B cells in follicular lymphomas and diffuse large B-cell lymphomas, neither in extranodal marginal zone lymphomas at non-cutaneous sites.^{5–7,18} In contrast to B-NHL, PD-1 is expressed by the neoplastic cells of several types of T-NHL, most notably angioimmunoblastic T-cell lymphoma (AITL), which is currently considered as the prototype of neoplasms derived from TFH cells.^{19,20} Regarding cutaneous T-cell lymphomas, PD-1, CXCL13 and BCL6, but –unlike AITL– not CD10, are consistently expressed by CD4⁺ small to medium-sized pleomorphic CTCL, but not or rarely by other types of primary cutaneous peripheral T-cell lymphoma, not otherwise specified, and not by primary cutaneous CD30-positive lymphoproliferative disorders.^{16,21} In recent studies of our group, PD-1 was almost without exception strongly expressed by the neoplastic T cells in Sézary syndrome, but uncommonly by the tumor cells in skin lesions of mycosis fungoides.¹⁷

In this study we also looked for the number and distribution of reactive PD-1⁺ T cells in CBCL and found that PD-1⁺ T cells were significantly more numerous in PCFCL than in PCMZL and PCLBCL–LT, which is consistent with the results of previous studies.^{12,13} In accordance, studies in nodal B-NHL showed significantly higher numbers of PD-1⁺ lymphocytes in follicular lymphomas than in diffuse large B-cell lymphomas.^{7,8} In PCFCL these PD-1⁺ T cells were not only located within germinal centers, but also outside germinal centers. Examination of serial sections

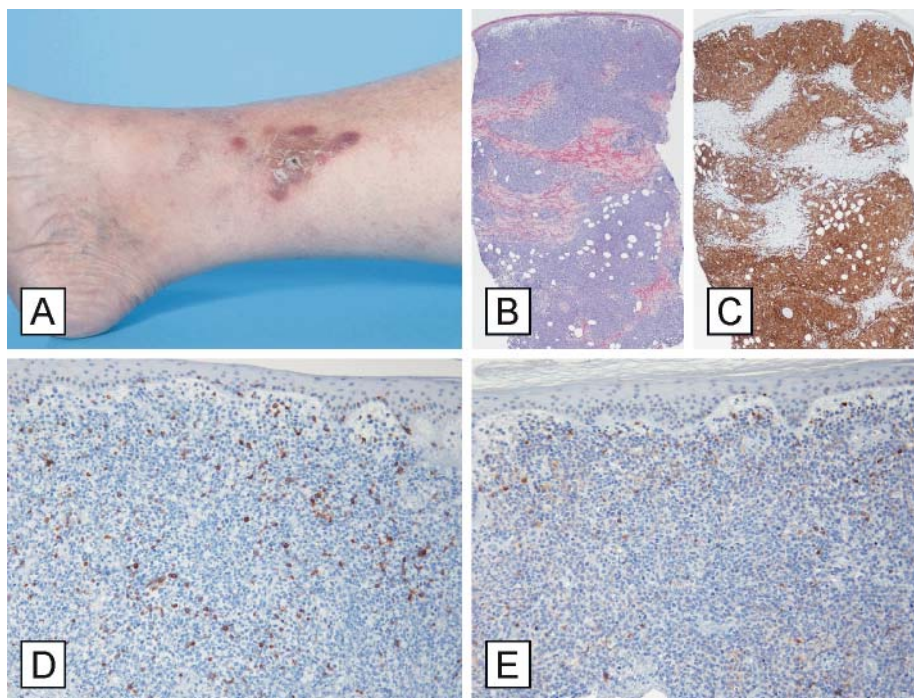


Figure 5. Histopathologic features of a primary cutaneous diffuse large B-cell lymphoma-leg type (PCLBCL-LT) patient. (A) Patient presenting with tumors on the medial side of the right ankle. (B) Hematoxylin-eosin staining of the lesion showing diffuse infiltrates of large neoplastic B cells, (C) which are CD79a positive. (D) The infiltrates contain few admixed reactive CD3⁺ T cells, of which (E) a small proportion showed programmed death-1 positivity. (B and C, original magnification x25; D and E, original magnification x100).

stained with follicular dendritic cell markers (CD21 and CD35) revealed that these areas with many PD-1⁺ T cells outside germinal centers still contained remnants of follicular dendritic cell networks, that were easily overlooked in routine hematoxylin-eosin stainings. A very similar distribution has been described in nodal follicular lymphomas.⁷⁻¹¹ In contrast, in PCMZL as well as the pseudo-B-cell lymphomas included as controls, PD-1⁺ T cells were preferentially localized within the germinal centers of reactive lymphoid follicles, and rarely present outside these follicles, which is in line with previous studies.^{12,13} The presence of many PD-1⁺ cells outside germinal centers may therefore be considered as an adjunct to differentiate PCFCL from pseudo B-cell lymphomas. Both in PCFCL and in PCMZL clusters of PD-1⁺ T cells, part of which were also CXCL13⁺, formed rosettes around large B cells, similar to TFH cells in reactive lymph nodes, as also reported in previous studies.^{12,13}

Several independent studies indicate that higher frequencies of intratumoral PD-1⁺ lymphocytes are associated with improved overall survival in nodal follicular

lymphomas, although in one study a relationship with poorer outcome was reported.⁷⁻¹⁰ In addition, the PD-1⁺ T cell abundance correlates with histological grade, as grade III lesions tend to contain fewer PD-1⁺ cells than grade I and II follicular lymphomas. In line with this, we found that PCFCL with a follicular or a follicular and diffuse growth pattern contained higher frequencies of PD-1⁺ T cells than PCFCL with a diffuse growth pattern. In contrast, in the study of Mitteldorf *et al.* no relation between PD-1⁺ cells and growth pattern was found, which may relate to the small patient groups studied.¹³ However, PD-1 expression has no prognostic significance in this group, as PCFCL has an excellent prognosis, irrespective of the growth pattern and the proportion of admixed (PD-1⁺) T cells.

For many years there has been discussion about the distinction between PCFCL with a diffuse growth pattern and PCLBCL–LT. In the WHO 2001 classification both were still classified as diffuse large B-cell lymphoma. However, studies in the last decade have firmly established that these represent distinct entities with a different clinical presentation, clinical behavior and prognosis, which are therefore classified separately in recent consensus classifications.^{14,22} In this study PCFCL with a diffuse growth pattern contained significantly more admixed PD-1⁺ T cells than PCLBCL–LT (50% versus 15%, respectively), suggesting that staining for PD-1 may serve as an adjunct diagnostic tool in difficult cases.

In conclusion, our results show that PD-1 is rarely expressed by the neoplastic B cells in CBCL. Moreover, apart from germinal centers of reactive lymphoid follicles in PCMZL, high numbers of PD-1⁺ T cells are particularly found in PCFCL. In particular, the presence of many PD-1⁺ T cells outside germinal centers may support a diagnosis of PCFCL.

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REFERENCE LIST

1. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* 2010;236:219-242.
2. Fife BT, Pauken KE. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. *Ann N Y Acad Sci* 2011;1217:45-59.
3. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011;29:621-663.
4. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* 2010;11:535-542.
5. Roncador G, Garcia Verdes-Montenegro JF, Tedoldi S et al. Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. *Haematologica* 2007;92:1059-1066.
6. Xerri L, Chetaille B, Serriari N et al. Programmed death 1 is a marker of angioimmunoblastic T-cell lymphoma and B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia. *Hum Pathol* 2008;39:1050-1058.
7. Muenst S, Hoeller S, Willi N, Dirnhofera S, Tzankov A. Diagnostic and prognostic utility of PD-1 in B cell lymphomas. *Dis Markers* 2010;29:47-53.
8. Carreras J, Lopez-Guillermo A, Roncador G et al. High numbers of tumor-infiltrating programmed cell death 1-positive regulatory lymphocytes are associated with improved overall survival in follicular lymphoma. *J Clin Oncol* 2009;27:1470-1476.
9. Wahlin BE, Aggarwal M, Montes-Moreno S et al. A unifying microenvironment model in follicular lymphoma: outcome is predicted by programmed death-1--positive, regulatory, cytotoxic, and helper T cells and macrophages. *Clin Cancer Res* 2010;16:637-650.
10. Richendollar BG, Pohlman B, Elson P, Hsi ED. Follicular programmed death 1-positive lymphocytes in the tumor microenvironment are an independent prognostic factor in follicular lymphoma. *Hum Pathol* 2011;42:552-557.
11. Alvaro T, Lejeune M, Salvado MT et al. Immunohistochemical patterns of reactive microenvironment are associated with clinicobiologic behavior in follicular lymphoma patients. *J Clin Oncol* 2006;24:5350-5357.
12. Fanoni D, Tavecchio S, Recalcati S et al. New monoclonal antibodies against B-cell antigens: possible new strategies for diagnosis of primary cutaneous B-cell lymphomas. *Immunol Lett* 2011;134:157-160.
13. Mitteldorf C, Bieri M, Wey N et al. Expression of PD-1 (CD279) in cutaneous B-cell lymphomas with correlation to lymphoma entities and biologic behaviour. *Br J Dermatol* 2013;169:1212-1218.
14. Willemze R, Jaffe ES, Burg G et al. WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005;105:3768-3785.
15. Koens L, Vermeer MH, Willemze R, Jansen PM. IgM expression on paraffin sections distinguishes primary cutaneous large B-cell lymphoma, leg type from primary cutaneous follicle center lymphoma. *Am J Surg Pathol* 2010;34:1043-1048.

16. Cetinozman F, Jansen PM, Willemze R. Expression of programmed death-1 in primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma, cutaneous pseudo-T-cell lymphoma, and other types of cutaneous T-cell lymphoma. *Am J Surg Pathol* 2012;36:109-116.
17. Cetinozman F, Jansen PM, Vermeer MH, Willemze R. Differential Expression of Programmed Death-1 (PD-1) in Sezary Syndrome and Mycosis Fungoides. *Arch Dermatol* 2012;148:1379-1385.
18. Grzywnowicz M, Zaleska J, Mertens D et al. Programmed death-1 and its ligand are novel immunotolerant molecules expressed on leukemic B cells in chronic lymphocytic leukemia. *PLoS One* 2012;7:e35178.
19. Dorfman DM, Brown JA, Shahsafaei A, Freeman GJ. Programmed death-1 (PD-1) is a marker of germinal center-associated T cells and angioimmunoblastic T-cell lymphoma. *Am J Surg Pathol* 2006;30:802-810.
20. Gaulard P, de Leval L. Follicular helper T cells: implications in neoplastic hematopathology. *Semin Diagn Pathol* 2011;28:202-213.
21. Rodriguez-Pinilla SM, Roncador G, Rodriguez-Peralto JL et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol* 2009;33:81-90.
22. Swerdlow SH, Campo E, Harris NL. WHO classification of tumors-haematopoietic and lymphoid tissues. WHO Press, Geneva, Switzerland 2008.

7

SUMMARY AND DISCUSSION

The studies presented in this thesis are focused on the expression of the molecule programmed death-1 (PD-1) in cutaneous lymphomas. The main goal was to determine the presence and distribution pattern of this molecule in different types of primary cutaneous T-cell lymphomas (CTCL) and cutaneous B-cell lymphomas (CBCL) in order to find out if PD-1 might be a useful biomarker to improve diagnosis, and possibly, to enable better classification of provisionally classified cutaneous lymphoma entities. In one chapter two additional markers were investigated, namely thymocyte selection-associated high mobility group box protein (TOX) and C-MYC. In this final chapter, the results described in this thesis are summarized and compared with the data described in the literature and conclusions are drawn.

PD-1 EXPRESSION AS DIAGNOSTIC MARKER FOR PRIMARY CUTANEOUS CD4⁺ SMALL/MEDIUM-SIZED PLEOMORPHIC T-CELL LYMPHOMA AND CUTANEOUS PSEUDO-T-CELL LYMPHOMA

In recent cutaneous lymphoma classifications [World Health Organization (WHO)-European Organization for Research and Treatment of Cancer 2005; WHO 2008], primary cutaneous CD4⁺ small/medium-sized pleomorphic T-cell lymphoma (PCSM-TCL) is a cutaneous T-cell lymphoma (CTCL) that has been included as a provisional entity.^{1,2} Histologically, these lymphomas show nodular-to-diffuse infiltrates with a predominance of CD3⁺, CD4⁺, CD8⁻, CD30⁻ small to medium-sized pleomorphic T cells and a small proportion (<30%) of large CD4⁺ pleomorphic T cells. In most cases there is a considerable admixture with small reactive CD8⁺ T cells and CD20⁺ B cells, including some blast cells, histiocytes, and in some cases plasma cells and eosinophils. Clinically, these PCSM-TCL characteristically present with a solitary plaque or tumor that is generally localized on the face or the upper trunk, and uncommonly with multiple papules, plaques, or tumors.^{1,3,4} In particular, patients presenting with a solitary skin lesion have an excellent prognosis.⁵ The clinical, histological and immunophenotypical features of these PCSM-TCL are strikingly similar to those described previously in so-called pseudo-T cell lymphomas.^{6,7} Demonstration of a T-cell clone and loss of pan-T-cell antigens are often used as diagnostic criteria for PCSM-TCL. However, using the Biomed-2 protocol, clonal TCR gene rearrangements are now found in most cases classified as pseudo-T-cell lymphoma and loss of pan-T-cell antigens is not observed in PCSM-TCL presenting with a solitary lesion (**Chapter 2**). Therefore, the relationship between PCSM-TCL and pseudo-T-cell lymphomas is still a matter of debate. More important, these PCSM-TCL/pseudo-T-cell lymphomas should be differentiated histologically from

other types of CTCL, in particular cutaneous peripheral T-cell lymphoma, not otherwise specified (PTCL–NOS) and tumor stage mycosis fungoides (MF).

In 2009, Rodriguez-Pinilla *et al.* reported that the large atypical CD4⁺ T cells in PCSM-TCL express PD-1, BCL6, and CXCL13.^{Ref 8} In **Chapter 2** we investigated expression of PD-1 and other TFH markers in skin biopsies of 26 cases classified as either PCSM-TCL or pseudo-T-cell lymphoma and skin biopsies obtained from patients with MF and a cutaneous PTCL–NOS. The primary goal of this study was to determine whether PD-1 could serve as a useful marker to differentiate between PCSM-TCL and these other types of CTCL. In all 26 PCSM-TCL/pseudo-T-cell lymphoma cases the medium-sized to large atypical T cells consistently expressed PD-1, which is in agreement with observations of more recent studies.^{9;10} In addition, serial sections showed that CXCL13 generally stained 50–75% of the PD-1⁺ cells, and BCL6 25–50% of the PD-1⁺ cells, suggesting that these tumor cells might have been originated from TFH cells. In contrast, PD-1 expression was found in only 2 of 16 PTCL–NOS and in only 2 of 21 cases of MF. Although not a specific marker, the presence of scattered or small clusters of medium-sized atypical CD4⁺ T cells expressing PD-1 and many admixed small reactive CD8⁺ T cells, CD20⁺ B cells and histiocytes, strongly suggests a diagnosis of PCSM-TCL/pseudo-T-cell lymphoma. This characteristic pattern was found in both cases classified initially as pseudo-T-cell lymphoma and cases classified as PCSM-TCL. Therefore, we and others⁴ feel that there is no reason to classify PCSM-TCL and pseudo-T-cell lymphoma separately. There is increasing doubt if such cases should be considered as a genuine malignant lymphoma and, small- to medium-sized pleomorphic T-cell nodules of undetermined significance and cutaneous CD4⁺ small-medium T-cell lymphoproliferation have been suggested as unifying terms for this condition (Beltraminelli *et al.*,⁴ and Chapter 2).

As all patients had an uneventful follow-up and that a skin relapse was only observed in a few cases, we suggest these cases should not be treated aggressively and no staging procedures should be performed. PCSM-TCL cases that do not meet the criteria described above are rare and should be fully staged. PCSM-TCL cases with rapidly growing multiple bulky tumors, a low percentage of admixed CD8⁺ T cells, and/or a high proliferative fraction, have been described to be at risk to develop progressive disease,⁵ but this study awaits further confirmation.

Recently, Battisella and colleagues described five patients with a CTCL, who presented with papules, nodules and/or infiltrated plaques not preceded by chronic patches or plaques, excluding MF.¹¹ Except for one patient with a solitary infiltrated plaque, who responded well to radiotherapy, the other cases were relatively resistant to current therapies for CTCL. Histologically, these cases showed either a non-epidermotropic subepidermal band-like or a diffuse dense dermal lymphoid infiltrate, containing about 50% CD3⁺ T cells and 50% CD20⁺ B cells. The infiltrates contained a subset of atypical medium to large CD4⁺ T-cells, which expressed TFH

markers PD-1, BCL6, CXCL13, CD10 or ICOS. Therefore, the authors suggested the term primary cutaneous TFH lymphomas for their cases. However, the relationship between these TFH-expressing CTCL and PCSM-TCL is at present unclear.

DIFFERENTIAL EXPRESSION OF PD-1 BY NEOPLASTIC CELLS IN SS AND MF

In our initial study described in Chapter 2, PD-1 expression was found in only a small proportion of MF cases (2 out of 21 cases), which contrasted with the results of earlier studies.¹²⁻¹⁴ Moreover, our results in MF also contrast with the high expression of PD-1 reported in skin and peripheral blood of patients with SS.^{13;15} This prompted us to investigate PD-1 expression in a large number of skin biopsies obtained from patients with SS and patients with MF, including cases with patch/plaque, tumor and erythrodermic stage of diseases and cases with a CD4⁺CD8⁻, CD4⁻CD8⁺ and CD4⁻CD8⁻ T-cell phenotype.

The results presented in **Chapter 3** showed that PD-1 was expressed by more than 50% of the neoplastic T cells in the skin of the majority (89%) of patients with SS. The association of PD-1 with Sézary cells is very strong. Even if a cut-off level of 75% was applied, still 81% of the SS patients were PD-1 positive. In line with our observation, Wada and colleagues found PD-1 expression by Sézary cells in 73% of skin biopsies of SS patients.¹³ In addition, Samimi and colleagues found increased PD-1 expression in the circulating neoplastic T cells in SS, but not in CD4⁺ peripheral blood T cells from patients with MF or healthy controls.¹⁵

Our data revealed that 25% to 50% of the PD-1⁺ neoplastic cells in SS co-expressed CXCL13 and BCL6, so it may be questioned whether Sézary cells originate from TFH cells and/or have functional characteristics of TFH cells. Picchio *et al.*¹⁶ found high expression of CXCL13 in skin lesions, lymph nodes, and peripheral blood of most patients with SS (over 80%), but only in a minority of patients with MF, which nicely parallels our observations. A derivation of Sézary cells from TFH cells seems unlikely, as the presence of B cells is uncommon in SS skin specimens and no relationship between PD-1 expression and the number of admixed B cells or plasma cells was found. Apparently, the simultaneous expression of TFH-cell markers PD-1, CXCL13, and BCL6 by a particular T cell does not necessarily mean that this cell is a TFH cell.

Consistent with results in chapter 2, the results in Chapter 3 show that PD-1 expression by >50% of the neoplastic T cells was found in a minority (only 13%) of the MF cases. We did not observe differences in PD-1 expression between the patch/plaque, tumor and erythrodermic cases. In addition, no differences between the CD4⁺CD8⁻, CD4⁻CD8⁺ and CD4⁻CD8⁻ T-cell phenotypes was found. These

Table 1. Expression of PD-1 in mycosis fungoides: comparison of the literature

	Total samples	PD-1 antibody (supplier)	Cut-off level for positivity	Number of positive cases	Percentage of positive cases	CD10 expression
Roncador <i>et al.</i> (2007)	9	Mouse monoclonal antibody; clone NAT105 (in-house generated)	Not indicated	5	56%	ND*
Wada <i>et al.</i> (2011)	30 #	Goat polyclonal antibody AF1086 (R&D Systems)	> 30%	15	50%	ND
Kantekure <i>et al.</i> (2011)	26	Mouse monoclonal antibody (Abcam); clone not specified (either NAT105 or J116)	> 25% > 50%	16 7	62% 27%	ND
Cetinozman <i>et al.</i> (2012)	60	Goat polyclonal antibody AF1086 (R&D Systems)	> 10% > 50%	12 8	20% 13%	negative
Park <i>et al.</i> (2014)	25	Mouse monoclonal antibody clone NAT105 (Cell Marque)	> 10%	21	84%	negative
Bosisio and Cerroni (2015)	32 †	Mouse monoclonal antibody (Abcam); clone not specified (either NAT105 or J116)	> 10% > 50%	13–17 9–16	62–85% 43–76%	1 biopsy >50%

*Abbreviations: ND, not determined; MF, mycosis fungoides

#30 biopsies obtained from 26 patients with MF

†32 biopsies obtained from 17 patients with MF

results conflict with other studies on PD-1 expression in MF, showing variable (27–100%) PD-1 expression by the neoplastic T cells in MF specimens.^{10;12-14;17} Despite six studies have been published on expression of PD-1 in MF (Table 1), no consensus is reached on this topic. The diversity of these observations may be explained by differences in cut-off levels of PD-1 positivity, antibodies and staining protocols (Table 1). Interestingly, 1 out of 8 E-MF cases showed PD-1 expression compared to 24 out of 27 cases of SS, which contributes to the debate on the relationship between both conditions.

RELATIONSHIP BETWEEN MF AND SS

The relationship between SS and MF is still a matter of debate as extensively discussed in **Chapter 3**. On the one hand, there is a general belief that SS is a leukemic phase or leukemic variant of MF because of the morphological atypical cells with cerebriform nuclei) and phenotypical (CD3⁺CD4⁺CD8⁻ T cells) similarities between both conditions, which resulted in the concept of CTCL.¹⁸ In addition, patients with MF may sometimes present with erythroderma and/or develop peripheral blood involvement during follow-up, while rare patients with SS

may develop skin tumors as in MF. On the other hand, SS and MF show obvious differences in clinical presentation and course, there are histologic, phenotypic, and genetic differences between both conditions.¹⁹⁻²⁶ The list of differences between SS and MF is still growing (summarized in Table 2) and multiple authors have suggested that SS and MF should be considered as distinct entities arising from different functional T-cell subsets. The almost consistent expression of PD-1 and CXCL13 in SS (Chapter 3 and Picchio *et al.*¹⁶) and the uncommon expression of these markers in MF provide additional support for the view that SS and MF should be considered as distinct lymphomas.

Table 2. Differences between Sézary syndrome and mycosis fungoides

Feature	Sézary syndrome	Mycosis fungoides
clinical presentation	erythroderma	patches, plaques, tumor
skin histology	mainly perivascular	epidermotropic (basal layer)
blood involvement	by definition	rare
lymph node histology	monotonous Sézary cells	dermatopathic lymphadenopathy + blast cell
CD4/CD8 phenotype	consistently CD4 ⁺ CD8 ⁻ T-cell phenotype	may also present with CD4 ⁻ CD8 ⁺ or CD4 ⁻ CD8 ⁻ phenotype
cell of origin	central memory T cell	effector memory T cell
loss pan T cell markers CD2, CD3, CD5	rare (even in patients with advanced disease) < 5% of patients	frequent (in particular in advanced stage of disease) > 50% of patients
PD-1 expression (cut-off > 50% positivity)	> 90% of patients	< 15% of patients

DIFFERENTIAL DIAGNOSIS BETWEEN SÉZARY SYNDROME AND ERYTHRODERMIC INFLAMMATORY DERMATOSES

Clinical and histological differentiation between SS and erythrodermic inflammatory dermatoses (EID) is one of the most challenging issues in dermatopathology. We found (Chapter 3) that PD-1 is expressed by Sézary cells in the vast majority of SS, representing the prototype of erythrodermic CTCL. We wondered whether PD-1 would be a biomarker to distinguish SS from EID histologically. In contrast to the almost consistent expression of PD-1 in SS (92% of cases), our immunohistochemical study (**Chapter 4**) of skin biopsies from EID patients revealed that PD-1 was

Table 3. Differences between Sézary syndrome and benign erythroderma

Feature	Sézary syndrome	Benign erythroderma
Blood involvement		
• T cell clonality	100%	uncommon
• CD4/CD8 ratio >10	90%	uncommon
Epidemotropism	extensive/Pautrier	few/moderate
Infiltrate density	moderate to extensive	sparse to moderate
Epidermal T cells	CD4 ⁺ T cells	mainly CD8 ⁺ T cells
Dermal T cells	CD4 >> CD8	CD4 ≥ CD8 or CD4 < CD8
Epidermal PD-1 ⁺ T cells	only CD4	CD8 >> CD4
Dermal PD-1 ⁺ T cells (cut-off > 50% positivity)	> 90%	< 15%
TOX expression (cut-off > 50% positivity)	> 85%	< 5%
Loss of CD7 expression (expression <20% of T cells)	50%	none

expressed by more than 50% infiltrating dermal CD3⁺ T cells in only 4 out of 30 cases (13%) While in SS PD-1 is mainly present by CD4⁺ neoplastic T cells, in EID PD-1 is predominantly expressed by CD8⁺ reactive dermal and epidermal T cells (Table 3). Although partial loss of CD7 expression can commonly be observed in both SS and EID, expression of CD7 by $\leq 20\%$ of the infiltrating T cells was only found in SS. Our results suggest that expression of PD-1 by more than 50% of CD4⁺ T cells and expression of CD7 by less than 20% of the skin-infiltrating T cells may be considered as valuable adjuncts in the differentiation between SS and EID.

Using the same cohort as in Chapter 4, in **Chapter 5** we investigated the diagnostic significance of two other markers TOX and C-MYC. Increased TOX expression by malignant CD4⁺ T cells has been found in MF and SS, but information on TOX expression in EID is lacking. In addition, C-MYC positivity has been demonstrated in a considerable number of infiltrating lymphoid cells in MF and SS, while there is no information on C-MYC expression in EID.²⁷⁻³¹ We demonstrated that TOX was strongly expressed by more than 50% of the neoplastic T cells in 13 of 15 SS patients (87%), while all EID cases showed weak nuclear staining (Table 3), showing TOX positivity between 11% and 50% of the T cells. Our results are consistent with recent studies also showing strong nuclear staining for TOX by atypical CD4⁺ T cells in skin^{28;31} and blood³⁰ of SS patients compared to benign inflammatory dermatoses and healthy controls. No significant difference in C-MYC expression between SS and EID was found. In both groups C-MYC was expressed by 11-25% of the skin-infiltrating T cells in about half of the cases. Kanavaros et

al. and Goswami *et al.* reported similar percentages of C-MYC positivity in SS patients.^{32,33} Strong expression of TOX, but not C-MYC, can be another useful adjunct in the differentiation between SS and EID.

In conclusion, in order to make a proper distinction between SS and EID the a combination of the following criteria seems to be relevant (Table 3): presence of clonality of the T cells in blood/skin, the CD4/CD8 ratio of infiltrating T cells in dermis and epidermis and in blood, the degree of epidermotropism, positivity of PD-1 and/or TOX by skin-infiltrating T cells and presence of loss of CD7 expression.

PD-1 EXPRESSION IN CUTANEOUS B-CELL LYMPHOMAS

Several studies investigated PD-1 expression in nodal B-cell non-Hodgkin lymphomas (B-NHLs). Except for small lymphocytic lymphoma and rare cases of nodal follicular B-cell lymphoma and diffuse large B-cell lymphoma, PD-1 is generally not present on neoplastic cells in B-NHLs.^{12,34,35} However, PD-1 is detectable on variable amounts of tumor infiltrating lymphocytes, in particular in follicular lymphoma.³⁵⁻³⁹ In most studies increased numbers of PD-1⁺ T cells in these nodal follicular lymphomas are associated with a significantly improved survival.³⁵⁻³⁸ As described in **Chapter 6**, we studied PD-1 expression in primary cutaneous B-cell lymphomas and found that this marker was absent on neoplastic B cells, but was present on variable amounts of reactive T cells. PD-1⁺ T cells were significantly more numerous in primary cutaneous follicle center lymphoma (PCFCL) than in primary cutaneous marginal zone lymphoma (PCMZL) and primary cutaneous diffuse large B-cell lymphoma, leg type (PCDLBCL-LT). In both PCFCL and PCMZL, as well as in pseudo B-cell lymphomas, clusters of PD-1⁺ T cells, part of which were also CXCL13⁺, formed rosettes around large B cells, similar to TFH cells in reactive lymph nodes. We also showed that in PCFCL these PD-1⁺ T cells were not only located within germinal centers, but also outside germinal centers. These results were consistent with recent studies in CBCL.^{40,41}

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The results of the studies presented in this thesis demonstrated that PD-1 can serve as a useful tool in the diagnosis of CTCL in particular in PCSM-TCL/pseudo T-cell lymphomas and SS. However, it should be recognized that PD-1 is not specific marker, as the neoplastic T cells in some cases of CTCL (such as MF and PTCL-NOS) and reactive T cells in both benign and malign skin diseases might also express

PD-1. The presence of scattered or clusters of PD-1 on T cells in combination with many admixed CD8⁺ T cells, B cells, macrophages is highly suggestive of PCSM-TCL/ pseudo T-cell lymphomas and facilitates differentiation from other types of CTCL, such as tumor stage MF or PTCL-NOS. PD-1 and TOX are strongly expressed in SS (90%) and are useful adjuncts to differentiate SS from benign erythroderma.

Apart from its diagnostic significance, PD-1 and PD-L1 expression in CTCL might also have therapeutic potential. The PD-1/PD-L1 pathway has emerged as an important mechanism by which tumors escape from the antitumor immune response. PD-1 is expressed by tumor-infiltrating lymphocytes in many different types of tumors,⁴² while high PD-L1 expression has been reported in many human cancers.⁴³ PD-L1 on the tumors binds to PD-1 on the tumor-infiltrating T lymphocytes causing reduction of the effector function and killing capacity of these T cells and consequently facilitating immune evasion of the tumors.⁴⁴ Ligation of PD-1 by PD-L1 can be obstructed with blocking monoclonal antibodies directed either to PD-1 or to PD-L1 thereby restoring the antitumor immunity. Recent clinical trials with antibodies targeting the PD-1/PD-L1 pathway in patients with cancer demonstrated promising antitumor responses.^{45,46} It was demonstrated that PD-1/PD-L1 blockade is most effective in tumors with high PD-L1 expression,^{47,48} and it is proposed that tumor-associated PD-L1 expression can be used as biomarker for selecting patients who will have the best survival benefit upon PD-1/PD-L1-pathway inhibitor therapy.

Considering the importance of the PD-1/PD-L1 pathway, the question arises whether this pathway can also serve as a potential target in patients with SS and MF. Kantekure *et al.* showed strong expression of PD-L1 by neoplastic large transformed T cells in MF.¹⁴ Whether the neoplastic T cells in SS — in addition to PD-1 — also express PD-L1 is at present unknown. Clinical trials investigating the efficacy of an anti-PD-1 monoclonal antibody in patients with relapsed or refractory MF and SS have just started.

REFERENCES

1. Willemze R, Jaffe ES, Burg G *et al.* WHO-EORTC classification for cutaneous lymphomas. *Blood* 2005;105:3768-3785.
2. Swerdlow SH, Campo E, Harris NL *et al.* WHO classification of tumors - Haematopoietic and lymphoid tissues, Geneva, Switzerland. WHO PRESS 2008.
3. Grogg KL, Jung S, Erickson LA, McClure RF, Dogan A. Primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma: a clonal T-cell lymphoproliferative disorder with indolent behavior. *Mod Pathol* 2008;21:708-715.

4. Beltraminelli H, Leinweber B, Kerl H, Cerroni L. Primary cutaneous CD4+ small-/medium-sized pleomorphic T-cell lymphoma: a cutaneous nodular proliferation of pleomorphic T lymphocytes of undetermined significance? A study of 136 cases. *Am J Dermatopathol* 2009;31:317-322.
5. Bekkenk MW, Vermeer MH, Jansen PM et al. Peripheral T-cell lymphomas unspecified presenting in the skin: analysis of prognostic factors in a group of 82 patients. *Blood* 2003;102:2213-2219.
6. Rijlaarsdam JU, Scheffer E, Meijer CJ, Willemze R. Cutaneous pseudo-T-cell lymphomas. A clinicopathologic study of 20 patients. *Cancer* 1992;69:717-724.
7. Rijlaarsdam JU, Willemze R. Cutaneous pseudolymphomas: classification and differential diagnosis. *Semin Dermatol* 1994;13:187-196.
8. Rodriguez Pinilla SM, Roncador G, Rodriguez-Peralto JL et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. *Am J Surg Pathol* 2009;33:81-90.
9. Ally MS, Prasad Hunasehally RY, Rodriguez-Justo M et al. Evaluation of follicular T-helper cells in primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma and dermatitis. *J Cutan Pathol* 2013;40:1006-1013.
10. Bosio FM, Cerroni L. Expression of T-follicular helper markers in sequential biopsies of progressive mycosis fungoides and other primary cutaneous T-cell lymphomas. *Am J Dermatopathol* 2015;37:115-121.
11. Battistella M, Beylot-Barry M, Bachelez H, Rivet J, Vergier B, Bagot M. Primary cutaneous follicular helper T-cell lymphoma: a new subtype of cutaneous T-cell lymphoma reported in a series of 5 cases. *Arch Dermatol* 2012;148:832-839.
12. Roncador G, Garcia Verdes-Montenegro JF, Tedoldi S et al. Expression of two markers of germinal center T cells (SAP and PD-1) in angioimmunoblastic T-cell lymphoma. *Haematologica* 2007;92:1059-1066.
13. Wada DA, Wilcox RA, Harrington SM, Kwon ED, Ansell SM, Comfere NI. Programmed death 1 is expressed in cutaneous infiltrates of mycosis fungoides and Sezary syndrome. *Am J Hematol* 2011;86:325-327.
14. Kantekure K, Yang Y, Raghunath P et al. Expression patterns of the immunosuppressive proteins PD-1/CD279 and PD-L1/CD274 at different stages of cutaneous T-cell lymphoma/mycosis fungoides. *Am J Dermatopathol* 2012;34:126-128.
15. Samimi S, Benoit B, Evans K et al. Increased programmed death-1 expression on CD4+ T cells in cutaneous T-cell lymphoma: implications for immune suppression. *Arch Dermatol* 2010;146:1382-1388.
16. Picchio MC, Scala E, Pomponi D et al. CXCL13 is highly produced by Sezary cells and enhances their migratory ability via a synergistic mechanism involving CCL19 and CCL21 chemokines. *Cancer Res* 2008;68:7137-7146.
17. Park JH, Han JH, Kang HY, Lee ES, Kim YC. Expression of follicular helper T-cell markers in primary cutaneous T-cell lymphoma. *Am J Dermatopathol* 2014;36:465-470.
18. Edelson RL. Cutaneous T cell lymphoma: mycosis fungoides, Sezary syndrome, and other variants. *J Am Acad Dermatol* 1980;2:89-106.

19. Willemze R, Scheffer E, Meijer CJ. Immunohistochemical studies using monoclonal antibodies on lymph nodes from patients with mycosis fungoides and Sezary's syndrome. *Am J Pathol* 1985;120:46-54.
20. Scheffer E, Meijer CJ, van Vloten WA, Willemze R. A histologic study of lymph nodes from patients with the Sezary syndrome. *Cancer* 1986;57:2375-2380.
21. Sentis HJ, Willemze R, Scheffer E. Histopathologic studies in Sezary syndrome and erythrodermic mycosis fungoides: a comparison with benign forms of erythroderma. *J Am Acad Dermatol* 1986;15:1217-1226.
22. Diwan AH, Prieto VG, Herling M, Duvic M, Jone D. Primary Sezary syndrome commonly shows low-grade cytologic atypia and an absence of epidermotropism. *Am J Clin Pathol* 2005;123:510-515.
23. Massone C, Crisman G, Kerl H, Cerroni L. The prognosis of early mycosis fungoides is not influenced by phenotype and T-cell clonality. *Br J Dermatol* 2008;159:881-886.
24. van Doorn R, van Kester MS, Dijkman R et al. Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. *Blood* 2009;113:127-136.
25. Campbell JJ, Clark RA, Watanabe R, Kupper TS. Sezary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. *Blood* 2010;116:767-771.
26. Clark RA, Watanabe R, Teague JE et al. Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients. *Sci Transl Med* 2012;4:117ra7.
27. Zhang Y, Wang Y, Yu R et al. Molecular markers of early-stage mycosis fungoides. *J Invest Dermatol* 2012;132:1698-1706.
28. McGirt LY, Adams CM, Baerenwald DA, Zwerner JP, Zic JA, Eischen CM. miR-223 regulates cell growth and targets proto-oncogenes in mycosis fungoides/cutaneous T-cell lymphoma. *J Invest Dermatol* 2014;134:1101-1107.
29. Huang Y, Litvinov IV, Wang Y et al. Thymocyte selection-associated high mobility group box gene (TOX) is aberrantly over-expressed in mycosis fungoides and correlates with poor prognosis. *Oncotarget* 2014;5:4418-4425.
30. Huang Y, Su MW, Jiang X, Zhou Y. Evidence of an oncogenic role of aberrant TOX activation in cutaneous T-cell lymphoma. *Blood* 2015;125:1435-1443.
31. Morimura S, Sugaya M, Suga H et al. TOX expression in different subtypes of cutaneous lymphoma. *Arch Dermatol Res* 2014;306:843-849.
32. Kanavaros P, Ioannidou D, Tzardi M et al. Mycosis fungoides: expression of C-myc p62 p53, bcl-2 and PCNA proteins and absence of association with Epstein-Barr virus. *Pathol Res Pract* 1994;190:767-774.
33. Goswami M, Duvic M, Dougherty A, Ni X. Increased Twist expression in advanced stage of mycosis fungoides and Sezary syndrome. *J Cutan Pathol* 2012;39:500-507.
34. Xerri L, Chetaille B, Serriari N et al. Programmed death 1 is a marker of angioimmunoblastic T-cell lymphoma and B-cell small lymphocytic lymphoma/chronic lymphocytic leukemia. *Hum Pathol* 2008;39:1050-1058.
35. Muenst S, Hoeller S, Willi N, Dirnhofera S, Tzankov A. Diagnostic and prognostic utility of PD-1 in B cell lymphomas. *Dis Markers* 2010;29:47-53.

36. Carreras J, Lopez-Guillermo A, Roncador G et al. High numbers of tumor-infiltrating programmed cell death 1-positive regulatory lymphocytes are associated with improved overall survival in follicular lymphoma. *J Clin Oncol* 2009;27:1470-1476.
37. Wahlin BE, Aggarwal M, Montes-Moreno S et al. A unifying microenvironment model in follicular lymphoma: outcome is predicted by programmed death-1--positive, regulatory, cytotoxic, and helper T cells and macrophages. *Clin Cancer Res* 2010;16:637-650.
38. Richendollar BG, Pohlman B, Elson P, Hsi ED. Follicular programmed death 1-positive lymphocytes in the tumor microenvironment are an independent prognostic factor in follicular lymphoma. *Hum Pathol* 2011;42:552-557.
39. Alvaro T, Lejeune M, Salvado MT et al. Immunohistochemical patterns of reactive microenvironment are associated with clinicobiologic behavior in follicular lymphoma patients. *J Clin Oncol* 2006;24:5350-5357.
40. Fanoni D, Tavecchio S, Recalcati S et al. New monoclonal antibodies against B-cell antigens: possible new strategies for diagnosis of primary cutaneous B-cell lymphomas. *Immunol Lett* 2011;134:157-160.
41. Mitteldorf C, Bieri M, Wey N et al. Expression of programmed death-1 (CD279) in primary cutaneous B-cell lymphomas with correlation to lymphoma entities and biological behaviour. *Br J Dermatol* 2013;169:1212-1218.
42. Ahmadzadeh M, Johnson LA, Heemskerk B et al. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 2009;114:1537-1544.
43. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. *Nat Rev Immunol* 2008;8:467-477.
44. Andorsky DJ, Yamada RE, Said J, Pinkus GS, Betting DJ, Timmerman JM. Programmed death ligand 1 is expressed by non-hodgkin lymphomas and inhibits the activity of tumor-associated T cells. *Clin Cancer Res* 2011;17:4232-4244.
45. Kyi C, Postow MA. Checkpoint blocking antibodies in cancer immunotherapy. *FEBS Lett* 2014;588:368-376.
46. Philips GK, Atkins M. Therapeutic uses of anti-PD-1 and anti-PD-L1 antibodies. *Int Immunol* 2015;27:39-46.
47. Powles T, Eder JP, Fine GD et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* 2014;515:558-562.
48. Herbst RS, Soria JC, Kowanetz M et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 2014;515:563-567.

8

APPENDIX

- SUMMARY IN DUTCH •
(NEDERLANDSE SAMENVATTING)
- LIST OF ABBREVIATIONS •
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SUMMARY IN DUTCH

(Nederlandse samenvatting)

Cutane lymfomen, oftewel huidlymfomen, zijn non-Hodgkin lymfomen die primair in de huid beginnen. Op het moment dat zij ontdekt worden, zijn er geen aanwijzingen dat het lymfoom elders in het lichaam voorkomt. Het is van groot belang om primaire cutane lymfomen te onderscheiden van lymfomen die in lymfeklieren zijn ontstaan en zich secundair manifesteren in de huid, omdat er tussen beide groepen grote verschillen bestaan in klinisch gedrag, prognose, therapie en reactie op behandeling. Cutane lymfomen zijn relatief zeldzaam. De geschatte jaarincidentie is 1:100.000 wat neer komt op circa 170 nieuwe patiënten per jaar in Nederland. Er bestaan diverse typen cutane lymfomen met een behoorlijk uiteenlopende relatieve frequentie (variërend van < 1% tot 45%) en ziektegerelateerde 5-jaarsoverleving (variërend van 0% tot 100%).

In de praktijk blijkt de diagnostiek van cutane lymfomen vaak lastig. Verschillende typen cutane lymfomen met een verschillend klinisch beloop en overlevingsverwachting kunnen eenzelfde histologisch beeld geven. Dit impliceert dat het voor een patholoog in veel gevallen onmogelijk is om alleen op basis van het histologisch beeld een definitieve diagnose te stellen. Combinatie van de klinische observatie en de histopathologie is essentieel om te komen tot een correcte diagnose. Het stellen van een goede diagnose is van belang om de patiënt de meest geschikte therapie te kunnen geven en te voorkomen dat onnodig agressief behandeld wordt. Een correcte diagnose maakt het ook mogelijk om relevante informatie en een reële prognose te kunnen geven aan de patiënt. Hoewel in de praktijk de meeste cutane lymfomen geassocieerd kunnen worden volgens de classificatie van de World Health Organization – European Organization for Research and Treatment of Cancer (WHO-EORTC), blijkt het in sommige gevallen zeer lastig om tot een definitieve diagnose te komen. Het is daarom van grote betekenis om te kunnen beschikken over aanvullende diagnostische biomarkers waarmee een duidelijk onderscheid kan worden gemaakt tussen de verschillende soorten cutane lymfomen.

Het molecuul programmed death-1 (PD-1) heeft de afgelopen jaren veel aandacht gekregen van onderzoekers op het gebied van de tumorimmunologie. PD-1 vervult een belangrijke rol in de negatieve regulatie van de cellulaire immuunrespons. Opmerkelijk is dat de expressie van PD-1 een belangrijke biomarker blijkt te zijn voor het onderscheid tussen verschillende typen non-Hodgkin T-cellymfomen met extracutane lokalisaties. In de studies beschreven in dit proefschrift is onderzocht of PD-1 ook een bruikbare diagnostische marker zou kunnen zijn voor cutane lymfomen.

Het eerste deel van **Hoofdstuk 1** geeft een gedetailleerd overzicht over het eiwit PD-1 dat centraal staat in dit proefschrift. Gestart wordt met een uitvoerige beschrijving van de kenmerken van het molecuul PD-1 en de liganden PD-L1 en PD-L2 die aan PD-1 kunnen binden. Ook wordt aangegeven welke cellen deze membraaneiwitten tot expressie brengen. Zo blijkt bijvoorbeeld dat conventionele T cellen pas na activatie PD-1 tot expressie brengen op de celmembraan, terwijl zogenaamde folliculaire helper T cellen dit eiwit constitutief tot expressie brengen. In detail wordt het mechanisme beschreven hoe de functie van geactiveerde conventionele T cellen geremd wordt na interactie van PD-1 met ligand PD-L1 of PD-L2. Als gevolg van deze interactie vermindert de proliferatie van de T cellen, neemt de cytokine productie (onder andere IL-2, IFN- γ en TNF- α) af en wordt geprogrammeerde celdood (apoptosis) gestimuleerd. Geactiveerde T cellen, met name CD8-positieve cytotoxische T cellen, zijn essentieel voor het elimineren van tumorcellen. Als tumorcellen het ligand voor PD-1 tot expressie brengen zijn ze in staat om geactiveerde T cellen functioneel te remmen via interactie met PD-1, en op deze wijze zich te verweren tegen aanvallen van PD-1-positieve tumorinfiltrerende T cellen die de tumor proberen te killen. Inmiddels is aangetoond dat blokkade van deze PD-1/PD-L1 interactie een veelbelovende behandeling is voor verschillende soorten kanker.

Het tweede deel van **Hoofdstuk 1** beschrijft de expressie van PD-1 in nodale non-Hodgkin lymfomen en het belang van dit molecuul bij de diagnose. Tenslotte worden de klinische en histopathologische kenmerken van verschillende typen primaire cutane lymfomen uitvoerig beschreven. De heterogene groep van cutane lymfomen kan onderverdeeld worden in twee hoofdgroepen: circa 75–80% wordt gevormd door cutane T-cellymfomen (CTCL) en 20–25% door cutane B-cellymfomen (CBCL). Beide groepen kunnen weer verder onderverdeeld worden in verschillende typen CTCL en CBCL. Het blijkt dat kennis over PD-1 expressie bij de verschillende typen cutane lymfomen gering of afwezig is.

In de in **Hoofdstuk 2** beschreven studie is onderzocht of PD-1 een bruikbare diagnostische marker is waarmee verschillende typen CTCL van elkaar onderscheiden kunnen worden. Het is belangrijk dat het primair cutaan CD4⁺ klein/middelgrootcellig pleomorf T-cellymfoom (PCSM-TCL), dat een uitstekende prognose heeft, onderscheiden wordt van andere typen CTCL, in het bijzonder het primair cutaan perifeer T-cellymfoom, not otherwise specified (PTCL–NOS) en tumorstadium mycosis fungoides (MF), omdat ze naast een compleet verschillende prognose ook sterk verschillen in geschikte behandelingsmethoden. Naast PD-1 is ook de expressie van BCL6, CXCL13 en CD10 bepaald om een mogelijke relatie met folliculaire helper T cellen te bepalen, welke co-expressie van genoemde 4 markers hebben. We vonden bij alle 26 patiënten met PCSM-TCL of met een cutaan pseudo-T-cellymfoom, dat de atypische T cellen zowel PD-1 als BCL6 en

CXCL13 tot expressie brachten, maar geen CD10. Expressie van PD-1 werd slechts bij 2 van de 21 patiënten met MF en bij 2 van de 16 patiënten met een PTCL-NOS gevonden. Samenvattend kan worden gesteld dat PD-1 expressie door atypische cellen kenmerkend is voor PCSM-TCL en cutane pseudo-T-cellymfoom en dat PD-1 niet of nauwelijks tot expressie wordt gebracht bij andere typen CTCL. Deze studie toont aan dat PD-1 een bruikbare aanvullende marker is bij de diagnostiek van PCSM-TCL, en tevens dat de atypische T cellen in PCSM-TCL en cutane pseudo-T-cellymfoom fenotypisch lijken op folliculaire helper T cellen. Verder ondersteunt onze studie de visie dat PCSM-TCL en cutane pseudo-T-cellymfomen, die zich met een solitaire laesie presenteren niet apart geclassificeerd moeten worden maar samen één entiteit vormen.

De studie beschreven in hoofdstuk 2 liet zien dat in slechts 2 van de 21 MF patiënten PD-1 expressie kon worden gedetecteerd in de huidbiopsieën op neoplastische cellen. Dit resultaat verschilt van gepubliceerde studies van andere onderzoeksgroepen, die rapporteerden dat PD-1 expressie gewoonlijk kan worden waargenomen in het merendeel van de MF patiënten en ook in patiënten met Sézary syndroom (SS). Sommigen beschouwen SS als een leukemische fase van MF vanwege overlap in een aantal kenmerken, maar hierover bestaat geen consensus. Naar aanleiding van de tegenstrijdige waarnemingen omtrent PD-1 expressie bij MF patiënten hebben we een studie uitgevoerd met een groot cohort MF patiënten en includeerden ook patiënten met erythrodermatisch MF en SS. Deze studie is beschreven in **Hoofdstuk 3**. We vonden dat PD-1 tot expressie werd gebracht door meer dan 50% van de neoplastische T cellen bij 8 van de 60 MF patiënten (13%). Bij erythrodermatisch MF werd PD-1 expressie in slechts 1 van de 8 patiënten (12%) aangetoond. In tegenstelling tot de situatie bij (erythrodermatische) MF, vonden we een sterke PD-1 expressie door meer dan 50% van de neoplastische T cellen bij 24 van de 27 SS patiënten (89%). Deze studie bevestigt onze eerdere bevindingen (hoofdstuk 2) dat PD-1 positiviteit slechts bij een laag percentage MF kan worden aangetoond. Een waarschijnlijke verklaring voor de tegengestelde waarnemingen van andere onderzoeksgroepen is dat zij een ander drempelwaarde voor PD-1 positiviteit gehanteerd hebben. Volgens onze definitie was er pas sprake van positiviteit als meer dan 50% van de neoplastische T cellen PD-1 kleuring liet zien, terwijl andere onderzoeksgroepen hun samples al positief beschouwden als 25% van de neoplastische T cellen PD-1 positief waren. In sommige studies was de drempelwaarde niet gedefinieerd. Onze studie toont aan dat MF en SS differentiële PD-1 expressie hebben en deze bevinding ondersteunt de visie dat MF en SS verschillende entiteiten zijn.

Het is bekend dat het histopathologisch onderscheid tussen SS en een erythrodermie op basis van een goedaardige aandoening extreem moeilijk kan zijn. Wij hebben aangetoond dat PD-1 sterk tot expressie komt in huidbiopten van

SS patiënten, maar informatie over PD-1 expressie in goedaardige vormen van erythrodermie ontbrak. In de studie beschreven in **Hoofdstuk 4** is de expressie van PD-1 onderzocht in huidbipten van 30 patiënten met verschillende typen erythrodermatische dermatitis (12 gevallen met een idiopathische erythrodermie, 10 met een atopische erythrodermie, 6 met een psoriatische erythrodermie en 2 met een paraneoplastische erythrodermie) en vergeleken met de expressie in huidbiopsieën van 25 SS patiënten uit een eerdere studie. We vonden dat PD-1 tot expressie werd gebracht door meer dan 50% van het T-cel infiltraat bij 23 van de 25 SS patiënten (92%), terwijl dit bij slechts 4 van de 30 patiënten met een erythrodermatische dermatitis het geval was (13%). Opvallend was dat PD-1 expressie aanwezig was op CD4⁺ neoplastische T cellen bij SS, maar voornamelijk op dermale en epidermale CD8⁺ T cellen bij erythrodermatische dermatitis. Expressie van CD7 door $\leq 20\%$ van het T-cel infiltraat werd alleen gevonden in SS huidcoupes en in geen enkel preparaat van de 30 patiënten met een goedaardige vorm van erythrodermie. Onze studie toont aan dat PD-1 een bruikbare marker is bij de diagnostiek van een patient met een erythrodermie: indien in een huidbiopsie van een patiënt met een erythrodermie aangetoond wordt dat PD-1 tot expressie komt bij meer dan 50% van de CD4⁺ T cellen en/of CD7 bij $\leq 20\%$ van het T-cel infiltraat, dan is dit een sterke aanwijzing voor de diagnose SS.

In de studie in **Hoofdstuk 5** maakten we gebruik van een deel van dezelfde patiëntcohorten die ook werden gebruikt in het voorafgaande hoofdstuk. Het doel van deze studie was om te onderzoeken of de markers thymocyte selection-associated high mobility group box protein (TOX, een chromatine-geassocieerd eiwit) en transcriptiefactor C-MYC bruikbaar zijn als aanvullende diagnostische markers om SS te onderscheiden van goedaardige vormen van erythrodermie. Het was al bekend dat beide markers tot expressie komen in MF en SS, maar het was nog onbekend in hoeverre deze markers tot expressie komen bij een erythrodermatische dermatitis. We vonden dat TOX sterk in de kern tot expressie werd gebracht door meer dan 50% van de T cellen bij 13 van de 15 SS patiënten (87%), terwijl TOX zwak tot expressie werd gebracht bij minder dan 50% (mediaan 25%) van het T-cel infiltraat bij de erythrodermatische dermatitis patiënten. De cohorten SS en goedaardige erythroderma lieten geen verschil zien in C-MYC expressie. In beide groepen was de expressie C-MYC laag en varieerde tussen $< 10\%$ en 25%. Onze studie toont aan dat TOX expressie door meer dan 50% van het T-cel infiltraat een bruikbaar hulpmiddel is bij het onderscheid tussen SS en een goedaardige vorm van erythrodermie, terwijl C-MYC onbruikbaar is voor dit onderscheid.

Er zijn diverse publicaties over PD-1 expressie in nodale B-cel non-Hodgkin lymfomen. In het algemeen werd gevonden dat PD-1 niet aanwezig is op de neoplastische B cellen, maar wel (in wisselende hoeveelheden), op tumorinfiltrerende T cellen. In sommige publicaties werd zelfs gesuggereerd dat de aanwezigheid van

verhoogde aantallen PD-1⁺ T cellen geassocieerd is met een betere overleving. Informatie over PD-1 expressie in de verschillende typen CBCL was nagenoeg afwezig. In de studie beschreven in **Hoofdstuk 6** hebben we daarom de expressie en distributie van PD-1 op neoplastische B cellen en reactieve T cellen in primaire CBCL onderzocht: 10 patiënten met primair cutaan marginale zone lymfoom (PCMZL), 18 met primair cutaan follikelcentrumcellymfoom (PCFCL) en 12 met primair cutaan diffuus grootcellig B-cellymfoom, beentype (PCDLBCL-LT). We vonden dat neoplastische B cellen negatief waren voor PD-1 behalve 2 gevallen met PCDLBCL-LT. De frequentie PD-1⁺ T cellen in huidcoupes van PCFCL, PCMZL en PCDLBCL-LT patiënten was respectievelijk 20%, 10% en 3% van het totale aantal infiltraatcellen en respectievelijk 60%, 20% en 15% van het totaal aantal infiltraat T cellen. De frequentie PD-1⁺ T cellen in huidcoupes van PCFCL patiënten was significant hoger dan in PCMZL en PCDLBCL-LT. Onze studie toont aan dat PD-1 zelden tot expressie komt op neoplastische cellen in CBCL. Tevens suggereert onze studie dat aanwezigheid van hoge percentages PD-1⁺ T cellen, in het bijzonder wanneer deze gelokaliseerd zijn buiten het kiemcentrum, een ondersteuning is voor de diagnose PCFCL. We hebben geen aanwijzing gevonden dat het aantal PD-1⁺ T cellen in huidcoupes van CBCL een prognostische waarde heeft.

LIST OF ABBREVIATIONS

aa	amino acid
AITL	angioimmunoblastic T-cell lymphoma
ALCL	anaplastic large cell lymphoma
BCL6	transcription factor B-cell lymphoma 6
C-ALCL	cutaneous anaplastic large cell lymphoma
CBCL	cutaneous B-cell lymphoma
CLL	chronic lymphocytic leukemia
CTCL	cutaneous T-cell lymphoma
CXCL13	chemokine C-X-C motif ligand 13
CXCR5	chemokine C-X-C motif receptor 5
EID	erythrodermic inflammatory dermatosis
E-MF	erythrodermic mycosis fungoides
EORTC	European Organization for Research and Treatment of Cancer
Ig	immunoglobulin
IgC	immunoglobulin constant region
IgV	immunoglobulin variable region
IL	interleukin
ITIM	immunoreceptor tyrosine–based inhibitory motif
ITSM	immunoreceptor tyrosine–based switch motif
LPD	lymphoproliferative disorders
MF	mycosis fungoides
NHL	non-Hodgkin lymphoma
PCFCL	primary cutaneous follicle center lymphoma
PCLBCL–LT	primary cutaneous diffuse large B-cell lymphoma, leg type
PCSM–TCL	primary cutaneous CD4 ⁺ small/medium-sized pleomorphic T-cell lymphomas
PCMZL	primary cutaneous marginal zone lymphoma
PD-1	programmed death-1
PD-L	programmed death-1 ligand
PTCL–NOS	peripheral T-cell lymphoma, not otherwise specified
SS	Sézary syndrome
TCR	T-cell receptor
TFH	follicular helper T cell
TOX	thymocyte selection-associated high mobility group box protein
Treg	regulatory T cell
WHO	World Health Organization

LIST OF PUBLICATIONS

Kuskonmaz B, Gocer S, Ersoy-Evans S, **Çetinözman F**, Cetin M, Uckan D. Hyperacute graft-vs.-host disease after related HLA-identical umbilical cord blood transplantation *Pediatr Transplantation*. 2007; 11 (7): 818–820.

Çetinözman F, Ersoy-Evans S, Karaduman A. Otoimmün Progesteron Dermatiti: Olgu Sunumu ve Literatürün Gözden Geçirilmesi. *Turkderm*. 2009; 43 (3): 122–125.

Çetinözman F, Jansen PM, Willemze R. Expression of programmed death-1 in primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma, cutaneous pseudo-T-cell lymphoma, and other types of cutaneous T-cell lymphoma. *Am J Surg Pathol*. 2012; 36 (1): 109–116.

Çetinözman F, Aksoy DY, Elçin G, Yıldız BO. Insulin sensitivity, androgens and isotretinoin therapy in women with severe acne. *J Dermatolog Treat*. 2014; 25 (2): 119–122.

Çetinözman F, Jansen PM, Vermeer MH, Willemze R. Differential expression of programmed death-1 (PD-1) in Sézary syndrome and mycosis fungoides. *Arch Dermatol*. 2012; 148 (12): 1379–1385.

Çetinözman F, Koens L, Jansen PM, Willemze R. Programmed death-1 expression in cutaneous B-cell lymphoma. *J Cutan Pathol*. 2014; 41 (1): 14–21.

Çetinözman F, Jansen PM, Willemze R. Expression of programmed death-1 in skin biopsies of benign inflammatory vs. lymphomatous erythroderma. *Br J Dermatol*. 2014; 171 (3): 499–504.

Cinar N, **Çetinözman F**, Aksoy DY, Elcin G, Yıldız BO. Comparison of adrenocortical steroidogenesis in women with post-adolescent severe acne and polycystic ovary syndrome. *J Eur Acad Dermatol Venereol*. 2015; 29 (5): 875–880.

Bakar Ö, Sahin S, **Çetinözman F**, Willemze R, Tezcanli E, Çetin ED. Tumor-stage mycosis fungoides of the vulva successfully treated with local low-dose radiotherapy. *Dermatol Ther*. 2015; 28 (1):36–39.

Boonk SE, **Çetinözman F**, Vermeer MH, Jansen PM, Willemze R. Differential expression of TOX by skin-infiltrating T cells in Sézary syndrome and erythrodermic dermatitis. *J Cutan Pathol*. 2015; 42 (9): 604–609.

Çetinözman F, Jansen P, Willemze R. Clear cell acanthoma on the dorsum of the hand. *J Eur Acad Dermatol Venereol*. 2016; 30 (2): 378–380.

CURRICULUM VITAE

Fatoş Çetinözman was born on the 31st of August of 1979 in Izmir, Turkey. After finishing high school in her home town in 1996, she decided to become a physician, like her father who was a pediatrician. She moved to Ankara to study medicine at the Ankara University Faculty of Medicine and graduated in June 2003. During the following year she followed a pathology residency program at the same university. In November 2004 she started her dermatology residency training at the Hacettepe University Faculty of Medicine in Ankara under the supervision of Prof. dr. Nilgün Atakan. In February 2010 she accomplished her five-year dermatology residency and obtained her certification. As Fatoş had special interest in cutaneous lymphoma, she organized an internship in the department of dermatology at the Leiden University Medical Center (LUMC) under the supervision of Prof. dr. Rein Willemze, an internationally acknowledged expert in this field. At the end of March 2010 she moved to The Netherlands and started an internship project on programmed death-1 (PD-1) expression in cutaneous lymphoma in April 2010. The project became very successful and was officially admitted to the LUMC doctorate program in May 2012. The results of the doctoral study on PD-1 are described in this thesis. During her doctoral studies Fatoş was awarded with a one-year grant from the Turkish Society of Dermatology.

Concomitant with her doctoral studies, Fatoş started the assessment procedure (comprising of a series of exams testing general and professional knowledge and skills) for recognition and registration of her medical university diploma in The Netherlands. All exams have been finished and she is currently in anticipation of the decision of the Commission for Foreign Healthcare Graduates on behalf of the Ministry of Health, Welfare and Sport. Upon being acknowledged and listed in the Dutch Healthcare Professionals (BIG) register, she will start a residency period in the department of dermatology at the Leiden University Medical Center (LUMC) under the supervision of Prof. dr. Rein Willemze in order to have her dermatologist certificate acknowledged in The Netherlands as well. Her ultimate goal is to have an academic career, combining clinic and scientific research, and having a special interest in cutaneous tumors, with the aim to improve diagnostics and therapy.

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