Malignant T Cells Exhibit CD45 Resistant Stat3 Activation and Proliferation in Cutaneous T-Cell Lymphoma

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Abstract: CD45 is a protein tyrosine phosphatase, which is well-known for regulating antigen receptor signalling in T and B cells *via* its effect on Src kinases. It has recently been shown that CD45 can also dephosphorylate Janus kinases (Jaks) and thereby regulate Signal transducer and activator of transcription (Stat) activation and cytokine-induced proliferation in lymphocytes. Consequently, CD45 dysregulation could be implicated in aberrant Jak/Stat activation and proliferation in lymphoproliferative diseases. Despite high expression of the CD45 ligand, Galectin-1, in skin lesions from cutaneous T-cell lymphoma (CTCL), the malignant T cells exhibit constitutive activation of the Jak3/Stat3 signalling pathway and uncontrolled proliferation. We show that CD45 expression is down-regulated on malignant T cells when compared to non-malignant T cells established from CTCL skin lesions. Moreover, CD45 cross-linking does not suppress the constitutive activation of Stat3 in the malignant T cells and there is no correlation between the level of activated Stat3 and the level of CD45 expression on the malignant T cells. Furthermore, in contrast to non-malignant T cells, the malignant T cells are protected against CD45-mediated inhibition of proliferation. In conclusion, our data suggest that CD45 dysregulation might play a role in the aberrant proliferation and Jak3/Stat3 activation in CTCL.

keywoards: CD45, T cell lymphoma, CTCL, STAT, galectin.

INTRODUCTION

Cutaneous T-cell lymphomas (CTCLs) are the most frequent primary lymphomas of the skin [1]. They comprise a wide spectrum of heterogeneous lymphoproliferative disorders characterised by clonal accumulation of neoplastic T lymphocytes in the epidermis. Mycosis fungoides (MF) is the most common representative of CTCL [1,2]. The etiology is unknown but it has been shown that the Janus kinase 3 (Jak3)/Signal transducer and activator of transcription 3 (Stat3) [3] pathway is constitutively active in tumour cell lines obtained from independent skin biopsies and peripheral blood of patients suffering from CTCL [4-6]. Importantly, evidence that the Jak3/Stat3 pathway is constitutively active *in vivo* has also been provided [7,8]. Inhibitors of Jak3 block the constitutive activation of Stat3 and inhibit the proliferation of the tumour cells [4,5,9]. Furthermore, a dominant negative form of Stat3 blocks spontaneous cytokine production and triggers apoptosis in the malignant T cells [7,10]. Collectively, these findings suggest that Jak3/Stat3 activation plays a critical role in the tumorigenesis of CTCL. However, the molecular mechanisms underlying the aberrant Jak3/Stat3 activation remain unknown.

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In non-malignant T cells, Stat activation is only transient and strictly controlled by positive and negative regulators. An important class of negative regulators of Stat activation are protein tyrosine phosphatases (PTPs). Some PTPs dephosphorylate and inactivate Jaks, whereas others dephosphorylate specific tyrosine residues in cytokine /growth factor receptors, while yet others inactivate Stats through direct dephosphorylation of critical tyrosine residues [11]. Hence, abnormal expression of PTPs is likely to be involved in the constitutive Stat activation. Accordingly, it has been shown that malignant T cells from CTCL patients often have a deficient expression of the PTP SHP-1 due to hypermethylation of the SHP-1 promoter [12,13]. Interestingly, treatment of the malignant T cells with methylation inhibitors restored the SHP-1 expression and decreased the constitutive phosphorylation of Jak3 [12]. However, not all CTCL cells/patients have deficient expression of SHP-1 suggesting a deficient function of other negative regulators of Jak3/Stat3 signalling [14].

CD45, also known as leukocyte-common antigen (LCA), is a transmembrane PTP expressed on all nucleated cells in the haematopoietic system and it is one of the most abundant glycoproteins on the surface of lymphoid cells [15]. It has long been recognised that CD45 has an important positive regulatory effect on T and B cell antigen receptor mediated signalling through its ability to dephosphorylate negative

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regulatory tyrosine residues on Src kinases [16]. Recently, an unexpected function of CD45 was identified: CD45 is a Jak phosphatase that negatively regulates cytokine receptor signalling [17]. Consequently, dysregulation of CD45 could be implicated in Jak/Stat hyper-activity and abnormal regulation of cytokine-induced proliferation in lymphoproliferative diseases. Galectin-1, a member of the β-galactoside binding protein family, is a natural ligand of CD45 and is normally expressed in thymus, spleen, lymph nodes and bone marrow [18-20]. It has previously been shown that Galectin-1 can inhibit the proliferation and induce apoptosis in human T lymphocytes [21,22]. However, despite abundant expression of Galectin-1 in CTCL skin lesions [23,24], the malignant T cells exhibit constitutive activation of the Jak3/Stat3 signalling pathway and uncontrolled growth. Therefore, we decided to investigate the possible role of CD45 with respect to the aberrant Stat3 activation and proliferation of the malignant T cells in CTCL.

MATERIALS AND METHODOLOGY

Cell Lines

The malignant T-cell line MyLa2000 and the non-malignant T-cell line MyLa1928 were established from a plaque biopsy of a patient diagnosed with MF [25]. The malignant T-cell lines PB-1, 2A and 2B were established from a patient with progressive CTCL [26]. The Jurkat T-cell line, J-Tag, has been described elsewhere [27]. The $CD4^+$ human antigen (Ag) specific T-cell lines and peripheral blood lymphocytes (PBLs) derived from healthy donors have been described and characterized previously [28-31].

Flow Cytometry

For CD45 staining, $1x10^6$ cells were harvested and washed in FACS buffer (PBS, 5% FBS, 0.1% sodium azide). Then, they were stained with phycoerythrin (PE) conjugated anti-CD45 (clone T29/33 which reacts with all CD45 isoforms) or PE conjugated isotype control (mouse IgG2b) antibodies (Abs) from Leinco (St. Louis, MO, USA) for 30 minutes at 4°C in the dark. After final washing, the cells were resuspended in FACS buffer and analysed on FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). For intracellular phospho-Stat3 staining, $1x10^6$ cells were harvested and washed in PBS. Next, the cells were fixed for 10 minutes in 2% paraformaldehyde at 37°C and permabilized in 90% ice-cold methanol for 30 minutes. Finally, the cells were washed in FACS buffer and incubated with Alexa flour 488 conjugated anti-phospho-Stat3(Y705) (4/P-STAT3 from Becton Dickinson) or Alexa flour 488 conjugated isotype control (mouse IgG2a from Leinco) Abs for 1 hour at room temperature in the dark. After thorough washing, the cells were resuspendend in FACS buffer and analysed on FACScan.

CD45 Cross-Linking and Western Blotting

Twelve-well culture plates were pre-coated with 400 µL media or rabbit anti-mouse Abs (40 µg/mL) for 16 hours at 4°C. Subsequently, the plates were washed with PBS and incubated with 400µL of media, 15 µg/mL mouse anti-CD45 (clone HI30 which reacts with all CD45 isoforms) (Becton Dickinson) or 15 µg/mL isotype control (Leinco) Abs for 2 hours. After washing, $1x10^6$ cells were added per well and incubated for 90 minutes at 37 degrees in a final volume of 1.2 mL. Then, the cells were lysed and the total cell lysates analyzed by western blotting as previously described [32]. Abs used for western blotting were anti-Erk1/2, anti-Actin, anti-CD45 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-phospho-Stat3(Y705) (Nanotools Denzlingen, Germany).

Proliferation Assays

Assays were performed in 96-well round bottom tissue culture plates essentially as described previously [33]. In brief, the culture plates were pre-coated overnight with 50 µL rabbit anti-mouse Abs (40 µg/mL) at 4°C and washed extensively in PBS. Subsequently, 50 µL of media, 10 µg/mL anti-CD28 mAbs (9.3) [31] or 10 µg/mL anti-CD45 mAbs (Leinco) were added to the wells as given and incubated for 2 hours at 37°C. Next, MyLa2000 ($2.5x10^3$), Myla1928 ($2.5x10^4$) and Ag T cells ($2.5x10^4$) were added to the wells. Following incubation for 3 hours at 37°C, media with or without IL-2 (10^3 U/mL) was added to a final volume of 200 µL. Finally, the cells were incubated for 120 hours and [^{3}H]thymidine (Amersham) (1 µCi/well) was added 24 hours before harvest. The [^{3}H]thymidine incorporation was measured in a scintillation counter and the results were expressed as mean counts per minute (Cpm) from triplicate cultures. Likewise, Jurkat or MyLa2000 T cells were cultured in 96-well round bottom tissue culture plates with or without 3.5 µM Galectin-1 before determination of thymidine incorporation.

RESULTS

CD45 Expression is Down-Regulated on Malignant T Cells when Compared to Non-Malignant T Cells Derived from CTCL

Initially, we measured the expression of CD45 on malignant and non-malignant skin T cells derived from a CTCL plaque stage biopsy of the same patient. Interestingly, we found that the CD45 expression was strongly down-regulated on the malignant T cells when compared with the non-malignant T cells (Fig. **1A**). We also examined the expression of CD45 in the three malignant T cells (PB-1, 2A, 2B) established from another patient with CTCL. The PB-1 T-cell line is from a relatively early, clinically indolent disease stage, whereas the 2A and 2B T-cell lines are established from later and more aggressive disease stages [26]. As seen in Fig. (**1B**), the expression of CD45 in PB-1 cells was similar to that in peripheral blood lymphocytes (PBL) and PBLs treated with phytohaemagglutinin (PHA). In contrast, the expression of CD45 was severely down-regulated in 2A and 2B cells when compared to that of PBLs and PB-1 cells (Fig. **1B**) suggesting that CD45 expression is down-regulated during progression from indolent to aggressive disease. Because deficient expression of the PTP SHP-1 in CTCL cell lines is caused by hypermethylation of the SHP-1 promoter [12], we treated the malignant T cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. However, this did not effect the CD45 expression (data not shown) indicating that down-regulation of CD45 expression is not caused by hypermethylation of the CD45 promoter.

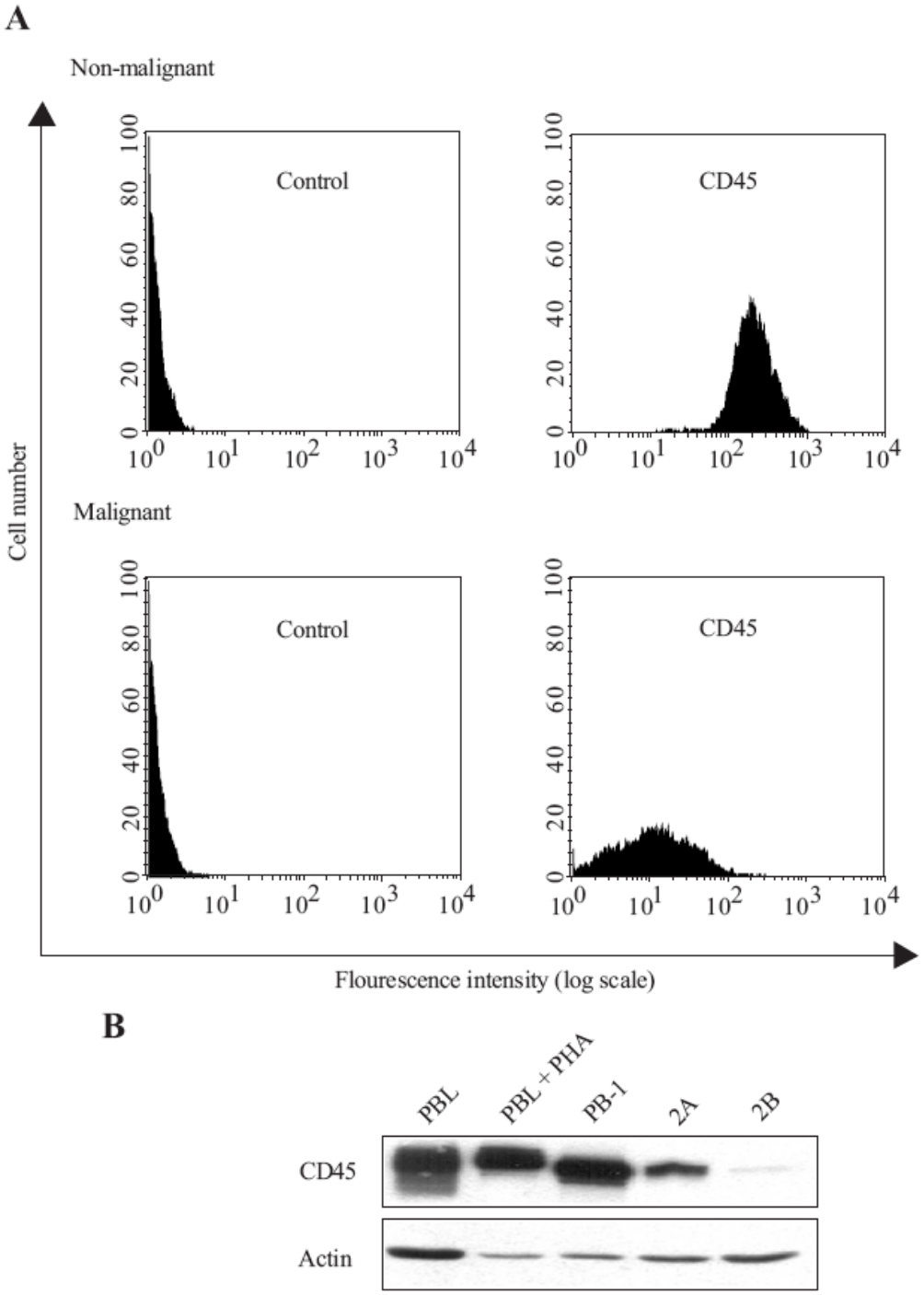

Fig. (1). Down-regulation of CD45 on malignant T cells when compared to non-malignant T cells. (**A**) Non-malignant (MyLa1928) and malignant (MyLa2000) T cells were stained with either a PE conjugated isotype control mAb (left) or an anti-CD45-PE mAb (right) and analysed by flow cytometry. (**B**) CD45 expression in peripheral blood lymphocytes (PBLs) treated with or without phytohaemagglutinin (PHA) and three malignant T cells derived from early- (PB-1) and late-stage (2A, 2B) CTCL lesions.

The Constitutive Activation of Stat3 is CD45 Resistant

It has previously been documented that CD45 cross-linking inhibits common γ-chain cytokine-mediated Stat3 phosphorylation [34]. Accordingly, we investigated if CD45 cross-linking could inhibit the constitutive activation of Stat3 in the malignant T cells. However, as seen in Fig. (**2A**), cross-linking of CD45 using an anti-CD45 mAb had no inhibitory effect on the level of phosphorylated Stat3 (pYStat3) in the malignant T cells. The expression of CD45 was relatively heterogeneous in the population of malignant T cells (Fig. **1**). Some cells were essentially CD45 negative ($CD45^{Neg}$) while others had low expression of CD45 ($CD45^{Low}$). Therefore, we examined whether CD45 expression was inversely correlated with pYStat3. As seen in Fig. (**2B**), the level of pYStat3 was similar in $CD45^{Neg}$ and $CD45^{Low}$ cells and, additionally, both resembled pYStat3 levels of the total cell population ($CD45^{Total}$). Thus, the low

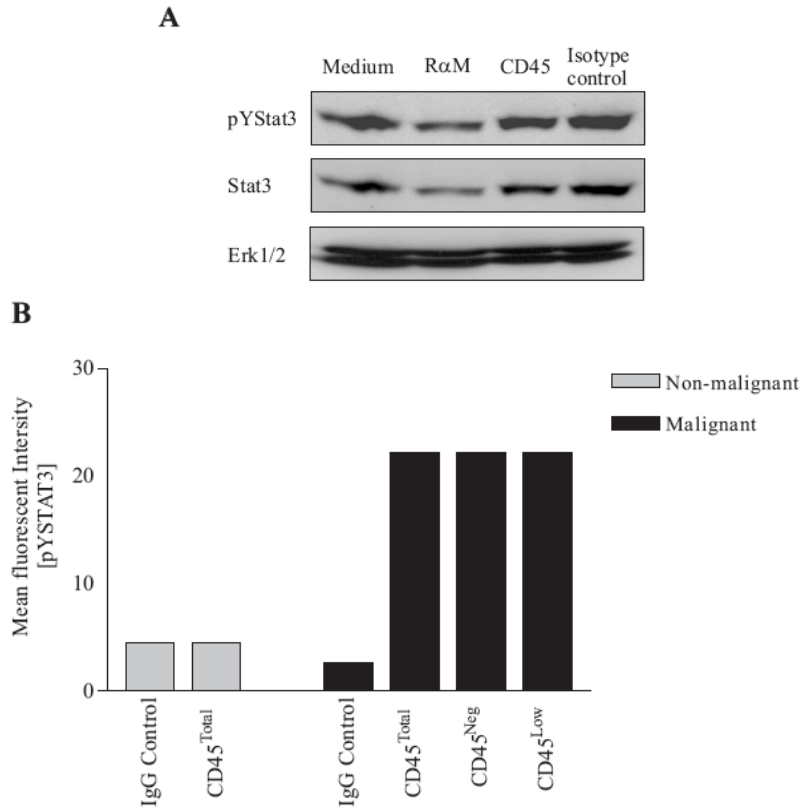

Fig. (2). Stat3 activation is CD45 resistant in the malignant T cells. (**A**) Malignant T cells (MyLa2000) were incubated for 90 minutes with medium or platebound rabbit anti-mouse (RαM), anti-CD45 or isotype control Abs. Then, the cells were lysed and the total cell lysates analysed by western blotting using anti-pY-Stat3, anti-Stat3 and anti-Erk-1/2 (loading control) Abs. (**B**) Non-malignant (MyLa1928) and malignant (MyLa2000) T cells were co-stained with Alexa flour 488 conjugated anti-pYStat3 and anti-CD45-PE mAbs or respective conjugated isotype control mAbs. The level of pYStat3 was measured by flow cytometry in the total cell population ($CD45^{Total}$) together with pYStat3 levels in the 10% highest ($CD45^{Low}$) and lowest ($CD45^{Neg}$) CD45 expressing cells.

levels of CD45 expressed on the malignant T cells were not sufficient to counteract the aberrant activity of Stat3. This prompted us to explore if higher levels of CD45 expression could suppress the constitutive activation of Stat3. Consequently, we transfected the malignant T cells with full length CD45, a truncated form of CD45 or an empty vector and subsequently tried to measure the level of pYStat3 in the transfected cells. However, the level of pYStat3 could not be reliably determined in cells transfected with full length CD45 as the majority (>95%) died shortly after transfection (data not shown). In contrast, transfection with truncated CD45 or an empty control vector did not induce cell death in the malignant T cells.

Malignant, But Not Non-Malignant, T Cells is Resistant to CD45 Mediated Inhibition of Proliferation

Recently, Blank *et al.* [34] reported that cross-linking of CD45 by mAbs inhibits common γ-chain cytokine mediated proliferation of activated human lymphoblasts. Therefore, we examined if CD45 cross-linking could inhibit the proliferation of the malignant T cells. As shown in Fig. (**3**), CD45 cross-linking by an immobilised anti-CD45 mAb mediated a profound inhibition of IL-2-induced proliferation of antigen-specific (Ag) T cells from healthy donors (Fig. **3A**) and likewise in non-malignant T cells from a CTCL patient (Fig. **3B**, left). The inhibition was specific, as an anti-CD28 mAb of same isotype had no inhibitory effect. Similar inhibition was observed with different CD45 mAbs indicating that this was a general feature of CD45 Abs (data not shown). Importantly, CD45 cross-linking had no effect on the proliferation of the malignant T cells (Fig. **3B**, right). Galectin-1 is a natural CD45 ligand which has been shown to be abundantly expressed in CTCL skin lesions [23,24]. It has previously been published that Galectin-1 can inhibit the proliferation of T cells [21], and as expected, Galectin-1 inhibited the proliferation of Jurkat T cells (Fig. **4**). However, in line with our previous findings, the malignant T cells from CTCL were less sensitive to Galectin-1 mediated suppression of proliferation (Fig. **4**). Collectively, these results indicate that CD45 down-regulation can protect the malignant T cells from CD45-mediated inhibition of proliferation.

DISCUSSION

In the present study, we show that CD45 expression is down-regulated on malignant T cells when compared to non-malignant T cells established from CTCL skin lesions. A finding which is in agreement with previous observations [35-37]. Furthermore, our results suggest that the down-regulation of CD45 expression primarily occurs during progression from indolent to more aggressive disease stages. Loss of CD45 expression has also been reported in other haematological malignancies such as acute lymphoblastic leukaemia [38] chronic lymphocytic leukemia [39] and

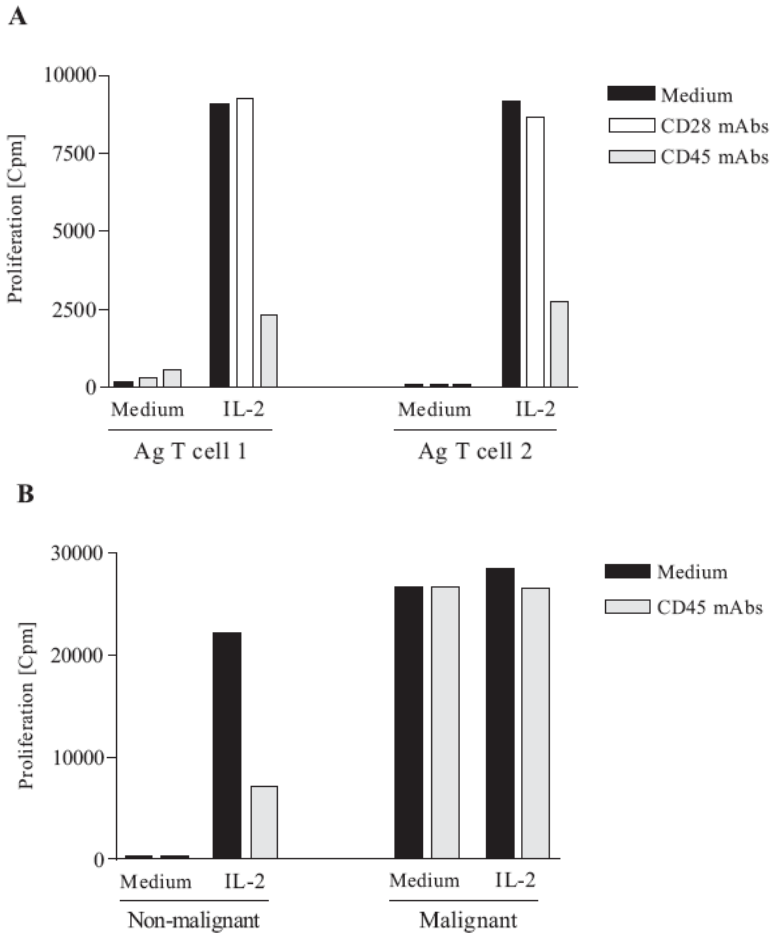

Fig. (3). CD45 cross-linking inhibits IL-2-induced proliferation of antigen specific T cells and non-malignant skin T cells but not malignant skin T cells. Round bottom tissue culture plates were coated with (**A**) anti-CD28 mAbs, (**A**, **B**) anti-CD45 mAbs or medium. Then (**A**) two different clones of antigen specific T cells (Ag T cell 1/2) from peripheral blood, (**B**, left) non-malignant skin T cells (MyLa1928) or (**B**, right) malignant T cells (MyLa2000) were added to the wells and incubated for 120 hours in culture medium with or without IL-2. [^{3}H]thymidine was added 24 hours before harvest and the incorporation measured in a scintillation counter. The results are expressed as mean counts per minute (cpm) from triplicate cultures.

Hodgkin's lymphoma [40] indicating that down-regulation of CD45 expression could play a role in the development and pathology of various haematological malignancies.

CD45 can inhibit Jak/Stat activation and cytokine-induced proliferation in lymphocytes [17,34]. Accordingly, we found that cross-linking of CD45 resulted in a profound inhibition of IL-2-induced proliferation of Ag T cells from healthy donors and non-malignant T cells from a CTCL patient. In contrast, CD45 cross-linking did not inhibit the proliferation of the malignant T cells. Likewise, CD45 cross-linking did not inhibit the constitutive activation of Stat3 in the malignant T cells and we found no correlation between the level of activated Stat3 and CD45 expression on the malignant T cells. Thus, our results suggest that the uncontrolled proliferation and aberrant Stat3 activation observed in the malignant T cells is resistant to CD45 cross-linking. This resistance could either be a consequence of the low CD45 expression, a dysfunction of CD45 or both. *In vivo*, CD45 cross-linking could be achieved through binding to one of its putative physiologic ligands, such as Galectin-1. Galectin-1 is highly expressed in CTCL skin lesions [23,24]

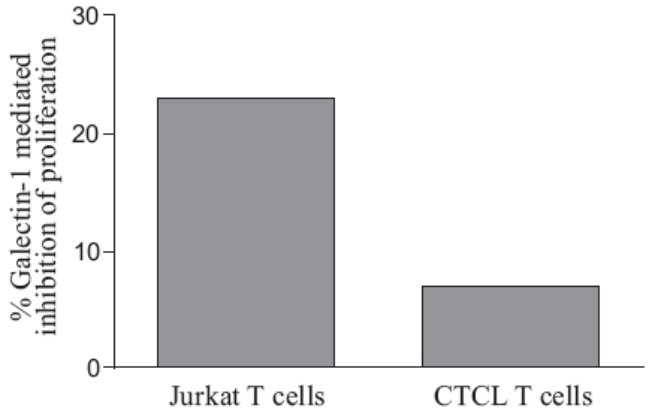

Fig. (4). CTCL cells exhibit increased resistance to Galectin-1 mediated inhibition of proliferation. Jurkat T cells or malignant T cells from CTCL (MyLa2000) were incubated for 48 hours with 3.5 μM of Galectin-1. [^{3}H]thymidine was added 24 hours before harvest and the incorporation measured in a scintillation counter. The results are expressed as mean counts per minute (cpm) from triplicate cultures.

indicating that CTCL cells are exposed to agents capable of cross-linking CD45 *in vivo*. Thus, CD45 resistance could give the malignant T cells a growth advantage by protecting them from CD45 mediated inhibition of proliferation. In line with this idea, Asosingh *et al*. [41] found that CD45 negative cells have a higher proliferative rate compared with CD45 positive cells in a murine model of Multiple Myeloma (MM). Furthermore, insulin-like growth factor 1 (IGF-1) has been shown to induce proliferation of human MM cells [42]. Induction of CD45 expression in such cells inhibit IGF-1 signalling [43] indicating that down-regulation of CD45 expression facilitates IGF-1 mediated proliferation of MM cells.

Inhibition of Stat3 activity has been shown to induce apoptosis in malignant T cells from CTCL [7]. Accordingly, we speculated that forced expression of CD45 in the malignant T cells would inhibit the activity of Stat3 and trigger apoptosis. In agreement with this hypothesis, the malignant T cells died shortly after transfection with full length CD45 but not a truncated form of CD45 or an empty vector. However, due to the quick induction of cell death, we were not experimentally able to resolve if the cell death was preceded by an inhibition of Stat3 activity. Therefore, we can't exclude that the cell death was triggered by events independently of CD45 function or by technicalities.

In conclusion, our results suggest that CD45 dysregulation might play a role in the aberrant proliferation and Jak3/Stat3 activation in CTCL.

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