Published in final edited form as:

Leuk Lymphoma. 2015 January ; 56(1): 186–193. doi:10.3109/10428194.2014.901511.

Involvement of tumor-associated macrophage activation *in vitro* during development of a novel mantle cell lymphoma cell line, PF-1, derived from a typical patient with relapsed disease

Lan V. Pham¹, Muychi T. Vang¹, Archito T. Tamayo¹, Gary Lu¹, Pramoda Challagundla¹, Jeffrey L. Jorgensen¹, Alex A. Rollo¹, Zhishuo Ou², Liang Zhang², Michael Wang², and Richard J. Ford¹

¹Department of Hematopathology, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

²Department of Lymphoma and Myeloma, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA

Abstract

Human mantle cell lymphoma (MCL) cell lines are scarce and have been only sporadically described and validated, and only a few have been thoroughly molecularly or genetically characterized. We describe here the successful establishment of a new MCL line, PF-1, with typical MCL characteristics. Culturing primary MCL cells *in vitro* initially gave rise to an essential generative microenvironment “niche” involving macrophages required for MCL growth, and eventually produced the PF-1 MCL cell line. Our analysis revealed that PF-1 is morphologically and genotypically nearly identical to the original tumor cells. The PF-1 MCL cell line that we have developed will be useful for *in vitro* and *in vivo* studies of MCL pathogenesis and therapeutics.

Keywords

Mantle cell lymphoma cell line; macrophages; *in vitro* microenvironment

Introduction

Mantle cell lymphoma (MCL) is one of the most challenging human cancers to treat, particularly among hematopoietic neoplasms, because it is typically one of the most therapeutically resistant forms of B-cell non-Hodgkin lymphoma (NHL-B) [1–4]. Currently considered an aggressive form of NHL-B [5], MCL was originally defined more specifically by the earlier Kiel classification system as a centrocytic or intermediate lymphocytic lymphoma with a distinct small B-cell non-Hodgkin lymphoma histotype that was often aggressive [6,7]. The presence of scattered epithelioid or “pink” histiocytes suggests a less aggressive variant referred to by other terms (i.e. centrocytic or intermediate). The MCL

Correspondence: Lan V. Pham, PhD, Department of Hematopathology, UT M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Unit 54, Houston, TX 77030, USA. Tel: 713-745-2391. lvpham@mdanderson.org.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahelthcare.com/lal.

immunophenotype is predominately “typical “ (small-cell) MCL, with rarer blastoid pleomorphic and less-aggressive mantle-zone variants.

Recently, isolated cases of more clinically indolent MCL were described to be associated with longer-term patient survival. Closer scrutiny of these cases of indolent MCL has suggested that most were non-nodal, were less systemically disseminated, tended to be leukemic, and showed certain immunophenotypic and genotypic characteristics [2]. Several reports of these cases of so-called *in situ* MCL, with or without SOX11 expression, suggested that rarer forms of indolent MCL can progress into more classic typical MCL cases *de novo* or on relapse [8–11]. Clearly, MCL is not the most monolithic pathologic entity as originally presumed, and an indolent course with the presence of pink histiocytes may point to different pathophysiological subtypes.

A major shortcoming of MCL research has been the relative lack of validated human or animal *in vitro* or *in vivo* model systems and experimental methodologies, which has hampered further understanding of the obscure ontogeny and complex pathophysiology required for definitive, translational studies in MCL [12]. Validated pathophysiological and experimental therapeutic models of this disease spectrum are urgently needed for essential translational research in MCL. MCL cell lines (typical or blastoid variants) are scarce and have been only sporadically described, and only a few have been thoroughly molecularly or genetically characterized [13–16].

We have been attempting to culture freshly explant MCL cells for extended periods. While this goal has been difficult to attain with established cell lines, we have had some surprising and interesting recent successes. As most investigators can attest, *in vitro* explanting of fresh MCL biopsy tissue or spleen leads to few viable MCL cells after short culture periods regardless of the media, sera and other factors. Recently, we have taken a different approach with MCL apheresis samples, and found that purified MCL cells indeed will not survive spontaneously *in vitro* or even in SCID (severe combined immune deficiency) mice. We have discovered, however, that freshly obtained MCL effusions and aphereses contain various cell types (or their precursors) that can give rise to an essential generative microenvironmental “ niche” that is required for *in vitro* MCL growth and survival (G/S) mechanisms. These studies have shown that MCL cell populations interact by spontaneously rosetting around autochthonous tumor macrophages, forming clonogenic cell clusters, and remain viable in culture for up to 2–4 months as the cellular complexity of the microenvironment increases. Macrophagic cell formation and division within the first 4 – 6 weeks are crucial components of the development of the critical microenvironmental niches. At that time, cell activation and mitotic figures appear more often, and putative “ transformed” MCL cells become more autonomous in their growth pattern and can be expanded *in vitro* eventually as a cell line.

Using this methodology, we developed a new typical MCL cell line, called PF-1, that phenotypically and cytogenetically resembles the initial MCL tumor clone. Here we describe the features of these PF-1 cells and compare them with primary MCL cells. Use of this validated MCL cell line should provide a much needed insight into the development and progression of this rare lymphoma.

Materials and methods

Cell culture

With informed consent from the patient, the collected primary cells were purified from an apheresis specimen by Ficoll centrifugation (Ficoll-Paque Plus; GE Healthcare, Life Sciences, Piscataway, NJ), washed in phosphate-buffered saline twice and resuspended in RPMI 1640 (Life Technologies, Grand Island, NY) containing 15% heat-inactivated fetal calf serum, 2 mM glutamine and 50 µg/mL gentamycin at a concentration of $5-10 \times 10^6$ cells/mL (40 mL) in 75 cm² flasks. Cultures were maintained at 37°C in a humidified incubator with a 5% CO₂ atmosphere. The medium was exchanged every 3 – 5 days depending on the cell growth rate. The cells were examined daily under an inverted microscope and counted weekly with a standard hemocytometer using trypan blue dye exclusion. No growth or stimulatory cytokines were added during the establishment of the PF-1 cell line.

Flow cytometry

Eight-color flow cytometry analysis was performed with FACS Canto II instruments (BD Biosciences, San Jose, CA) using commercially available reagents on patient samples collected in ethylenediaminetetraacetic acid or the cell line in culture medium. The cell population was gated using right-angle light scatter and CD45 expression. The panel of monoclonal antibodies used included those against CD3, CD4, CD5, CD8, CD10, CD19, CD20, CD22, CD23, CD34 CD43, CD44, CD200, FMC7, kappa light chain and lambda light chain. All the antibodies were from BD Biosciences except FMC7, which was obtained from Beckman-Coulter (Fullerton, CA). Data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA). Antigen expression was scored as positive if 30% of cells were brighter than a threshold set using a negative autofluorescence (empty channel) control.

Conventional cytogenetics analysis

After 72 h of culture, conventional G-banded karyotyping was performed with metaphase cells derived from the cultures using the protocol in the Clinical Cytogenetics Laboratory at the M. D. Anderson Cancer Center. Briefly, metaphase cells were obtained after hypotonic treatment and fixation with 3:1 methanol–acetic acid solution. Cell suspensions were dropped onto clean slides. G-banding was performed after the slides were dried at 60°C overnight. Fluorescence *in situ* hybridization (FISH) was performed on interphase nuclei from the cultures for routine cytogenetic study using *IGH/CCND1* dual-color, dual-fusion translocation probes (Abbott Molecular, Des Plaines, IL), as described previously. The cut-off to define a positive result for *IGH/CCND1* rearrangement was 0.03%. A total of 200 interphase cells were analyzed.

Short tandem repeat DNA fingerprinting

Genomic DNA was isolated from the original tumor or from the PF-1 cell line using a Qiagen DNA purification kit (Valencia, CA). DNA fingerprinting on lymphoma cells was performed by the Characterized Cell Line core facility at M. D. Anderson using the short

tandem repeat (STR) method. STR repeats are regions of microsatellite instability with defined tri- or tetrad-nucleotide repeats that are located throughout the chromosomes. Polymerase chain reaction (PCR) reactions using primers on non-repetitive flanking regions generate PCR products of different sizes based on the number of repeats in the region; the size of these products was determined by capillary electrophoresis. Extracted DNA was analyzed using the Power Plex 16HS System from Promega (Madison, WI). The relatedness of the original tumor and the PF-1 cell line was determined by comparing the STR loci profiles of the respective samples.

Epstein–Barr virus PCR amplification

Epstein–Barr virus (EBV) genotyping was performed by PCR using genomic DNA to amplify a common region of the EBNA2 gene. PCR was performed using the PCR Kit from Promega, with the following set of primers: EBNA-F: TGGAAACCCGTCCTCTC; EBNA-R: TAATGGCATAG-GTCCAATG. Cycling conditions were: 95°C 2 min, 40 cycles of 94°C 1 min, 60°C 90 s, 72°C 4 min, followed by 72°C for 10 min. The EBV-negative Mino and the EBV-positive Granta MCL cell lines were used as negative and positive controls, respectively. A non-specific band, approximately 300 bp, served as an internal control. PCR reactions were performed at least three times.

Western blot and confocal microscopic analysis

These methods have been previously described [17,18].

Results

Establishment of the PF-1 MCL cell line

Primary MCL cells were obtained from a patient diagnosed with MCL in October 2011. Initial chemotherapy treatment had consisted of eight cycles of frontline R-hyper-CVAD (rituximab with hyperfractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone) alternating with Rituxan, methotrexate and cytarabine (Ara-C) until March 2012. A complete remission was achieved and was followed by an autologous stem cell transplant in May 2012. The disease relapsed in August 2012. The patient then received salvage treatment with two cycles of RICE (rituximab with ifosfamide, carboplatin and etoposide) followed by one cycle of bendamustine, Rituxan, Velcade and Ara-C. An effusion developed that was tapped in December 2012 which yielded the primary MCL cells. During culture of the primary apheresis-derived MCL cells at various cell densities *in vitro*, the initial event that occurred in the tissue culture (within 7 days) was the formation of small clusters of tumor cells [Figure 1(A), day 7]. Within 14 days, a smaller subset of larger macrophage-like cells began to appear that behaved like normal adherent macrophages in that they engulfed dead or apoptotic MCL cells. Within 3–4 weeks, these large cells were fully developed and resembled activated macrophages. The MCL tumor cells tended to form a rosette, or cluster, around and adhere to these macrophages [Figure 1(B)]. The macrophages formed in culture coexisted with MCL tumor cells for up to 4 months. During this period, a small subset of MCL cells showed limited mitotic figures, suggesting that these cells were proliferating and becoming incipient immortalized MCL cells. When these

cells were weaned from the adherent macrophages, they began to expand autonomously and proliferate as an autonomous cell line.

Next, we examined whether these macrophagic-like cells expressed CD68, a typical marker for macrophages. In the initial sample [Figure 1(A), day 0], a small population of cells stained positive for CD68; we presumed that these cells were monocytes. Within 6–7 days, the CD68 + cells were larger and seemed to have engulfed multiple MCL cells, as indicated by the appearance of multiple nuclei inside the CD68 + cells. These CD68 + cells appeared to be fully activated macrophages within 14 days, and they were maintained in the tissue culture with the MCL cells. Because the MCL cells tended to cluster around the CD68 + cells [Figure 1(B), right panel], CD68 + cells might provide MCL cells with the key G/S signals upon which they depend to grow. When we cultured isolated MCL cells without the accessory cells, no CD68 + macrophagic cells were present after 14 days, and the MCL cells underwent rigorous apoptosis and eventually died out within 3–4 weeks [Figure 1(C)].

Growth and morphologic characteristics of PF-1 MCL cells

PF-1 cells grew singly in suspension in RPMI-1640 medium supplemented with 15% heat-inactivated fetal calf serum, and could be frozen in medium composed of 90% fetal calf serum and 10% dimethylsulfoxide (DMSO). Cells were optimally maintained at a density between 1 and 2×10^6 cells/mL and could be split 1:2 every 3–4 days. PF-1 cells appeared as small–medium lymphoid cells approximately 6–13 μ m in the longest diameter [Figure 2(A)]. The cells showed varying degrees of morphologic atypia with round–ovoid and sometimes notched or cleaved irregular nuclei contours with granular and condensed chromatin distribution. The morphology of PF-1 cells did not seem to change after 8 months in tissue culture [Figure 2(B)].

Immunophenotypic characterization of PF-1 MCL cells by flow cytometry

We performed flow cytometry on the original apheresis sample and on the established PF-1 cell line after 4 months of continuous culture. Representative flow cytometry histograms demonstrated that 99% of PF-1 cells were positive for both CD5 and CD19, and nearly all PF-1 cells (99.5%) were positive for kappa light chain and 0% were positive for lambda light chain [Figure 2(C)]. The immunophenotypic characterization of the primary MCL cells and the PF-1 cells is summarized in Table I. PF-1 cells expressed monotypic immunoglobulin kappa light chain, CD5, CD19, CD20 (dim), CD22 and CD43 and did not express CD3, CD4, CD8, CD10, CD14, CD23, CD56, CD200 or lambda light chain. While the primary cells exhibited a distinct subset (about 35%) positive for CD23, the PF-1 cells did not show CD23 expression significantly above background. The patient had been treated with R, an anti-CD20 antibody, which could have resulted in the selection of cells with low expression of CD20 in the primary cells, PF-1 cells or both. Apart from the dim CD20 expression, this immunoprofile for the PF-1 cell line corresponded to that of typical MCL cells.

Conventional cytogenetics and FISH analysis of PF-1 MCL cells

FISH analysis demonstrated that 95.5% of the primary MCL cells showed fusion signals for *IGH/CCND1* rearrangement, confirming the presence of t(11:14)(q13;q23). Conventional cytogenetic analysis of PF-1 cells revealed t(11:14)(q13;q32), the characteristic cytogenetic

signature for MCL, in addition to a very complex karyotype [Figure 3(A)]. A representative karyotype of PF-1 cells revealed a karyotype that is similar to but more complex than that of the primary MCL cells (Table II). The *in situ* hybridization technique was performed using an LSI *IGH/CCND1* dual-color, dual-fusion translocation probe to confirm the presence of t(11;14) in PF-1 cells. This probe hybridizes to band 11q13 (*CCND1*) and band 14q32 (*IGH*), and is useful for detecting t(11;14). A total of 200 interphases were analyzed. Nuclear fusion signals were observed in 100% of the interphases, indicating t(11;14)-positive cells [Figures 3(B) and 3(C)]. Western blot analysis also confirmed the protein expression of cyclin D1 in PF-1 cells in comparison to two other well-known typical MCL cell lines, Mino and Jeko [Figure 3(D)]. Besides cyclin D1, PF-1 cells also expressed CDK4 and p53 proteins [Figure 3(D)].

EBV status in PF-1 cells

EBV status in PF-1 cells was determined by PCR to detect the EBNA2 gene. EBV viral genomes were detected in the immortalized Granta MCL cell line that served as a positive control [Figure 3(E)]. However, they were not detected in the Mino MCL cell line (negative control) or PF-1 cell line [Figure 3(E)]. Our results indicated that PF-1 cells are EBV negative.

STR DNA fingerprinting analysis of primary MCL cells and PF-1 MCL cells

We also determined the authenticity of the PF-1 cell line using a multiplex STR DNA fingerprinting system, which allows for the detection of unique DNA fingerprints through the genotyping of 16 STR loci. The original MCL cells and the PF-1 cells shared 100% identity of the genotype, confirming the parentage of the latter (Table III). The PF-1 cell line profile did not match any profile in the current database at M. D. Anderson Cancer Center.

Discussion

A substantial number of preliminary studies with patient samples has made it clear that certain microenvironmental interactions are necessary for long-term *in vitro* culture of untransformed MCL cells. These usually indolent, neoplastic B cells do not independently grow spontaneously. Instead, they require active cellular interactions with microenvironmental cellular components that stimulate and sustain cell G/S. Perhaps not surprisingly, the monocyte/macrophage (MP) and related accessory cell lineages constitute the group of “nurse-like” cells derived from bone marrow and other lymphoid tissues that provide necessary microenvironmental cofactors *in vitro* and probably also *in vivo* [19–21]. Recent initial morphologic studies have usually noted that numerous scattered histiocytes (macrophages) were observed in at least 90% of MCL samples, whereas only 5–10% of samples of B-cell non-Hodgkin lymphoma contained similar macrophage populations [22,23]. Pink (epithelioid) histiocytes likely account for a significant component of the macrophages, but although they have been long recognized, these cells have not been studied in MCL in any detail. Virtually every other histotype of B-cell NHL and Hodgkin disease has now been shown to have significant macrophage component interactions that often show clinical significance [24]. More recent studies have shown that a high monocyte count at presentation in MCL can predict poor clinical outcomes [25,26]. The postulated mechanism

for this relationship is that high monocyte count is a surrogate biomarker of the tumor microenvironment, reflecting the recruited peripheral blood immunosuppressive monocytes by the tumor transforming into tumor-associated macrophages, leading to cancer progression.

Our studies [27,28] of large numbers of tumor cells from patients with non-nodal MCL have shown that when adequate numbers of unstimulated, putatively untransformed, and unseparated MCL cells in effusions (>90% morphologic or immunophenotypic) or leukemic cell populations are cultured, they progressively become, *in situ*, adherent MCL-rosetted MP aggregates. Cells harvested from effusions or leukemic cells predictably and reproducibly form in culture flasks, expanding in size, allowing for protracted MCL cell G/S. In contrast, MCL cells obtained from tumor tissues rigorously separated from accessory cells in culture do not proliferate, and usually die after less than 3 weeks. Assiduous attention to critical cell culture parameters (such as optimal cell density determination, viability demonstration and cell proliferation) and meticulous technical cellular maintenance methodology are required for successful long-term (> 2 months) MCL culturing and eventual immortalized cell line emergence and establishment.

The intrinsic value of being able to generate permanent immortalized MCL cell lines, particularly of less understood and more indolent forms of MCL, is obvious. Currently, it is unclear whether there is a difference between the PF-1 cell line and other existing MCL cell lines. PF-1 cells were derived from a typical patient with MCL with indolent phenotype (low Ki-67), and therefore this cell line most likely resembles other existing typical MCL cell lines, such as Mino and Jeko. The approach in developing the PF-1 cell line is unique, since the tumor microenvironment developed in tissue culture somewhat mimics what is seen in patients with MCL. Future research needs to delineate how MCL–MP interactions allow sustained MCL G/S and, in some cases, transformation or progression (or both) to clonally identical, highly proliferative and aggressive blastoid variants of MCL [27]. Our studies using low-grade or non-nodal MCL effusions or leukemic cell populations have shown that MCL–MP interactions are highly dynamic in most patients with lymphoma, with many MCL cells spontaneously binding to autochthonous macrophages that develop from circulating precursors, most likely of the monocyte or stromal cell “nurse-cell” lineage but possibly also from other cellular precursor sources that have been difficult to identify to date [29]. Developing such models should help to elucidate a clearer biologic and clinical image of the increasingly recognized multifaceted pathophysiology of different MCL subtypes in a more functional translational context, from the welter of microarray [30], genetic [31], microRNA [32], and so on, data to be mined, that have yet to provide a very clear picture regarding many important aspects of the MCL disease processes and pathophysiology [33].

In summary, we have described the establishment and detailed characterization of PF-1, a novel typical MCL cell line that resembles the original MCL cells from a patient. This new cell line will be a valuable tool for *in vitro* and *in vivo* studies of MCL pathogenesis and for translational drug screening.

Acknowledgments

We would like to thank the Farahi Family Foundation for supporting this study. STR DNA fingerprinting was done by the Cancer Center Support Grant-funded Characterized Cell Line core, NCI # CA016672. The authors thank Elizabeth L. Hess for editing the manuscript.

The University of Texas MD Anderson Cancer Center Lymphoma Tissue Bank is supported by the National Institutes of Health Lymphoma SPORE grant P50CA136411 and Fredrick B. Hagemeister Research Fund.

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Figure 1.

Representative images of monocyte/macrophage (MP) involvement in establishment of the PF-1 MCL cell line. (A) Hematoxylin and eosin (H&E) staining (left column), confocal microscopy analysis of CD68 + cells (red) (middle and right columns) of primary MCL cells in culture on days 0 (upper row), 7 (middle row) and 14 (bottom row). Topro3 was used to stain nuclei (blue). Original magnification, $\times 400$. (B) H&E staining (left panel) and confocal microscopy analysis of CD68 + cells (right panel), showing the interaction between macrophages and MCL cells after 4 weeks of cell culturing. (C) No macrophage transformation in purified MCL cells. Primary cells were left unpurified (left column) or purified using the B-cell separation kit (right column). Cells were cultured for 14 days and then stained for CD68 (red) and Topro3 (blue, nuclear marker). Arrows in the light field indicate tumor cells undergoing apoptosis.

Figure 2.

Morphologic and phenotypic features of PF-1 MCL cells. (A) Distribution of the size (longest diameter) of PF-1 MCL cells after 4 months of cell culturing. (B) Representative images of H&E-stained PF-1 MCL cells at different time points, 4 months (left panel) and 8 months (right panel) in tissue culture. (C) Representative immunophenotype flow cytometric histograms of PF-1 MCL cells. (Left) Immunostaining for both CD5 and CD19. (Right) Immunostaining for kappa and lambda light chains.

Figure 3.

Conventional cytogenetics and FISH analysis of PF-1 MCL cells. (A) Representative karyotype of PF-1 MCL cells. Arrows point to abnormalities listed in Table II. (B) FISH detection of t(11;14)(q13;q32) in PF-1 MCL cells. *IGH/CCND1* dual-color, dual-fusion translocation probes were used. Green: probe for the immunoglobulin heavy chain gene (14q32.2); red: probe for the *CCND1* gene (11q13); yellow: fusion gene signal. (C) Representative MapBack of FISH analysis showing t(11;14). (D) Western blot analysis of cyclin D1, CDK4 and p53 protein expressions in Mino and Jeko (two typical MCL cell lines) and PF-1 (new cell line) MCL cells. Actin served as an internal loading control. (E)

EBV status in PF-1 cells. PCR analysis for EBNA2 gene in Mino (negative control), Granta (positive control) and PF-1 MCL cells. M, molecular marker; NS, non-specific band that served as an internal control.

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Table I

Immunophenotype profile of primary MCL cells and PF-1 MCL cells as determined by flow cytometry.

Phenotype	Primary MCL cells	PF-1 MCL cells
CD5	+	+
CD10	–	–
CD19	+	+
CD20	+ (dim)	+ (dim)
Kappa light chain	+	+
Lambda light chain	–	–
CD23	+/-	–
CD43	+	+
CD34	–	–
CD200	–	–
CD22	+	+
CD3	–	–
CD4	–	–
CD8	–	–

MCL, mantle cell lymphoma; –, negative staining; +, positive staining; +/-, equivocal staining.

Table II

Clonal cytogenetic abnormalities in plasma cells from primary bone marrow sample and from PF-1 MCL cells.

Karyotype	
Primary MCL cells	PF-1 MCL cells
44~46, XY, der(2)inv(2) (p25q11.2)t(2;12)(q31;q13), del(4) (q31.3q35), add(9) (p22), add(10)(q24), t(11;14)(q13;q32), der(12) t(2;12)(p11.2;p11.2)t(2;12) (q31;q13), i(17)(q10) [cp8]	44~46, XY, del(1)(q32q44), der(2)inv(2)(p25q11.2)t(2;12) (q31;q13), del(4) (q31.3q35), add(5)(q34), -8, add(9)(q34), add(9)(p22), add(10)(q24), del(11) (p14), t(11;14)(q13;q32), der(12)t(2;12)(p11.2;p11.2)t(2;1) (q31;q13), +13, add(15)(p11.2), i(1)(q10)[cp20]

MCL, mantle cell lymphoma.

Table III

STR DNA fingerprinting of primary MCL cells and PF-1 MCL cells.

Sample	STR loci							
	AMEL	CSF1PO	D13S317	D16S539	D21S11	FGA	TH01	TPOX
Primary cells	X,Y	10,12	12	11	28,32.2	21,24	9,9.3	8,11
PF-1 cells	X,Y	10,12	12	11	28,32.2	21,24	9,9.3	8,11

STR, single tandem repeat; MCL, mantle cell lymphoma.