Inflammatory Monocytes Are a Reservoir for Merkel Cell Polyomavirus

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Merkel cell polyomavirus (MCPyV) is a recently discovered virus that is implicated in the oncogenesis of Merkel cell carcinoma (MCC). The route of dissemination and the reservoir(s) of MCPyV within the human body have not yet been identified. In this study we describe two patients with multiple MCPyV-positive inflammatory and neoplastic skin lesions at different anatomic sites. Patient 1 was suffering from psoriasis for many years and was diagnosed with MCC 7 years before this study. Patient 2 had developed numerous non-melanoma skin cancer lesions under post-transplant immunosuppression. In both patients, MCPyV DNA was detected in whole blood and in urine using PCR and direct sequencing of PCR products. When we analyzed different blood compartments, we found MCPyV exclusively in cell-free serum and in blood monocytes, but not in lymphocytes or granulocytes. Upon separate analysis of resident ($CD14^{lo}CD16^{+}$) and inflammatory ($CD14^{+}CD16^{-}$) monocytes, we detected MCPyV exclusively in inflammatory, but not in resident monocytes. Our findings raise the possibility that MCPyV persists in inflammatory monocytes and spreads along the migration routes of inflammatory monocytes. This points to intervention strategies to contain MCPyV. Moreover, blood or urine tests may serve as ancillary tests to confirm MCPyV infection in a clinical setting.

Journal of Investigative Dermatology (2010) **130,** 1146–1151; doi:10.1038/jid.2009.392; published online 17 December 2009

INTRODUCTION

Polyomaviruses are non-enveloped, double-stranded DNA viruses. They often persist as latent infections in the host, but are potentially oncogenic and may produce tumors upon reactivation. Merkel cell polyomavirus (MCPyV) is the most recently discovered human polyomavirus. It is monoclonally integrated into the genome of approximately 80% of human Merkel cell carcinomas (MCCs), which is a rare but aggressive neuroendocrine skin neoplasia (Feng *et al.*, 2008). It is suspected that MCPyV has a causal role in the oncogenesis of MCCs (Kassem *et al.*, 2008; Viscidi and Shah, 2008; Andres *et al.*, 2009; Duncavage *et al.*, 2009).

The natural transmission route of MCPyV and its reservoir(s) in the human body have not been established yet. Other polyomaviruses, such as BK virus (BKV) and JC virus (JCV), cause non-neoplastic inflammatory disorders of the urinary tract, are shed through the urine, and persist in kidney and lymphocytes (Markowitz *et al.*, 1993; Leung *et al.*, 2001; Zhong *et al.*, 2007). As a consequence, the potential routes for BKV transmission are oral, smear infection, blood transfusion, and organ transplantation, particularly renal allografts (Andrews *et al.*, 1988; Dolei *et al.*, 2000; Shah, 2000). For MCPyV, however, the most common routes of interindividual transmission cannot be appropriately addressed, until the reservoir of this virus in the body is known. If MCPyV were able to disseminate throughout the body through the circulation, the recipients of blood, blood products, and solid organ transplants might be at risk of getting exposed to this cancer-associated virus.

In this study we identified two patients with multiple skin lesions, including MCC, non-melanoma skin cancer, actinic keratosis, seborrheic keratosis, and psoriasis, who were tested positive for the presence of specific MCPyV DNA sequences. As we detected MCPyV DNA in both patients at various anatomic locations and associated with different diseases, we hypothesized that MCPyV might persist within and spread through the blood. Indeed, we detected MCPyV DNA in whole blood and in cell-free serum of both patients. Upon analysis of the phenotype of MCPyV-positive cells in the blood, inflammatory $CD14^{+}CD16^{-}$ monocytes stood out as the only reservoir of the virus in blood. As inflammatory monocytes accumulate within tumors and at sites of inflammation, our findings have important implications for the mechanisms of systemic dissemination of MCPyV within patients, and for the transmission between individuals.

RESULTS

Detection of MCPyV in an MCC patient

At 7 years before this study, a then 75-year-old Caucasian female who had been chronically suffering from psoriasis was

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Abbreviations: BKV, BK virus; JCV, JC virus; MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus; PBMC, peripheral blood mononuclear cell

Received 20 July 2009; revised 10 October 2009; accepted 23 October 2009; published online 17 December 2009

diagnosed with MCC. The tumor was surgically removed. The patient received intermittent psoralen UV-A light therapy, topical corticosteroids, and topical calcipotriol. She was never treated with retinoids or immunomodulators. When she presented again to our institution 7 years later, we excised two newly arisen skin lesions—one suspicious for seborrheic keratosis on her left thigh, and one of her psoriasis lesions on her left upper arm. Both diagnoses were confirmed histologically. Surprisingly, we detected MCPyV DNA in both lesions. Consequently, we analyzed the MCC from our archive that was excised 7 years before, together with a tumor-free normal skin biopsy from her left forearm that was taken at the same time. The old MCC lesion was harboring MCPyV DNA, whereas the normal skin was negative for the virus (Table 1).

To verify the specificity of the amplified products and to detect the possible occurrence of genomic variants, all MCPyV PCR products were sequenced. MCPyV sequences from the MCC, the seborrheic keratosis, and the psoriasis were identical. When compared with the nucleotide sequences available in the database of the National Center for Biotechnology Information, the identity was found to the MCV350 genome (gb|EU375803.1 Merkel cell polyomavirus isolate MCC350). Therefore, we concluded that the patient had been infected with a single clone of MCPyV that had persisted for at least 7 years in an unknown reservoir outside the MCC before it became associated with an inflammatory and a neoplastic skin lesion at distinct parts of the body.

As control cohorts for this isolated case, we screened psoriasis lesions from patients chronically suffering from psoriasis, but without MCC ($n=16$, of which nine were females, median age 46.5 years, range 18–90), and normal skin from healthy individuals ($n=7$, of which four were females, median age 40, range 19–78). In none of these control tissues, MCPyV DNA was detected using PCR.

Detection of MCPyV in a kidney transplant recipient

A 53-year-old Caucasian male recipient of a renal allograft under long-term immunosuppression (cyclosporine, azathioprine, and prednisolone) was regularly monitored for skin neoplasms in our institution for several years. Over time, he developed numerous actinic keratoses, seborrheic keratoses, and non-melanoma skin cancers. He harbored MCPyV DNA in approximately 30% of his skin lesions at various anatomic locations, e.g., in two out of three seborrheic keratoses (Table 2). The clonality of MCPyV sequences in this patient was confirmed by sequencing as well, and identity was found to the MCV350 genome (gb|EU375803.1 Merkel cell polyomavirus isolate MCC350). This, again, indicated MCPyV persistence outside of MCCs in yet unknown reservoirs.

The presence of MCPyV DNA at different anatomic locations in two different patients suggested that the virus got disseminated throughout the organism by a so far unidentified mechanism. We hypothesized that MCPyV could spread through the blood in either free form or in association with blood cells.

Detection of MCPyV DNA in peripheral blood and inflammatory monocytes of both MCPyV-positive patients

On the basis of this hypothesis, we set out to test whether MCPyV was detectable in the blood of these two patients. Indeed, whole-blood samples of both patients were positive for both large T and VP1 MCPyV sequences. Sequencing of the PCR products confirmed 100% homology to the MCV350 genome in both patients. When we analyzed corpuscular constituents of the blood and blood serum separately, we detected MCPyV in both fractions. This showed that the virus can occur freely or in cell-associated form. We also tested whole-blood samples from healthy individuals ($n=7$, of which five were females, median age 43, range 25–60) and from psoriasis patients without MCC ($n=6$, of which four were females, median age 51, range 35–81). In none of these patients, MCPyV DNA was detected in the blood.

Next, we analyzed which blood cells carry the virus. To this end, we first fractionated peripheral blood mononuclear cells (PBMCs) of both patients using FACS sorting, based on cell size (reflected as forward scatter) and granularity (reflected as side scatter, Figure 1a). MCPyV was exclusively associated with a population of large, non-granular cells, whereas small non-granular cells from the lymphocyte gate and small, granular cells from the granulocyte gate did not contain MCPyV sequences (Figure 1b).

As the MCPyV-positive signal was detected in the monocyte gate for both patients, we speculated that monocytes were specifically associated with MCPyV. Recently, two functional subsets of blood monocytes were characterized (Geissmann *et al.*, 2003): The $CD14^{lo}CD16^{+}$ ''resident'' monocytes have a longer half-life and the capacity to home to non-inflamed tissues. These cells may be identical with the

Table 1. Results of MCPyV PCRs for patient 1

Patient 1 diagnosis	Localization	Year	β	VP1	LT-1	LT-3
MCC	Forearm, left	2002	+	+	+	+
Normal skin	Forearm, left	2002	+	–	–	–
Seborrheic keratosis	Thigh, left	2009	+	+	+	+
Psoriasis	Upper arm, left	2009	+	+	+	+

Abbreviations: MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus.

Table 2. Results of MCPyV PCRs for patient 2

Patient 2 diagnosis	Number of lesions	Number of lesions positive for LT-3 or VP-1	Number of lesions positive for LT-3 and VP-1
Actinic keratosis	1	0	0
Bowen's disease (carcinoma *in situ*)	6	3	1
Seborrheic keratosis	3	2	1
Squamous cell carcinoma	8	0	0

Abbreviation: MCPyV, Merkel cell polyomavirus.

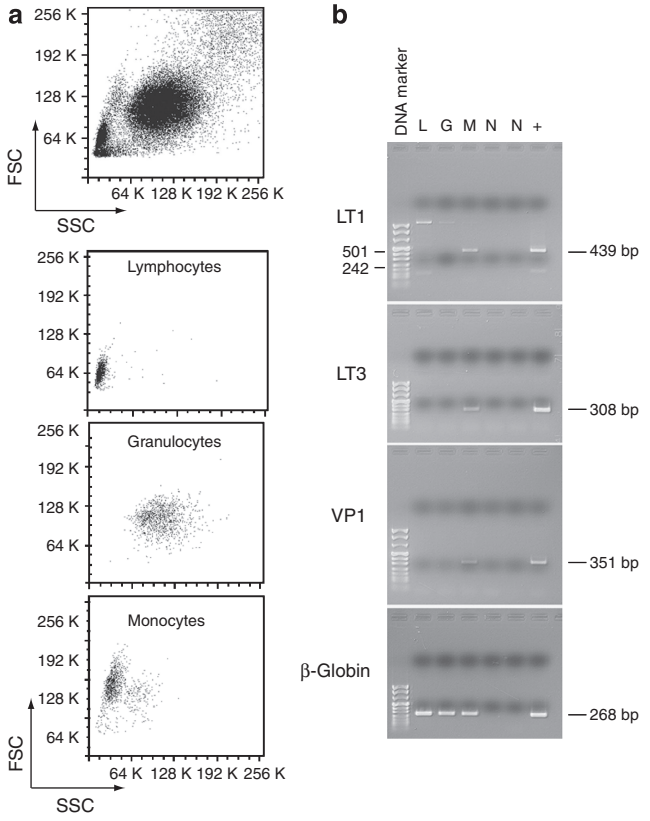

Figure 1. Detection of Merkel cell polyomavirus (MCPyV) in blood monocytes. (**a**) Whole blood of both patients was fractionated by FACS sorting, based on cell size (forward scatter, FSC) and granularity (side scatter, SSC). DNA was extracted from L, M, and G. (**b**) PCR analysis of these three populations revealed positive MCPyV signals in only monocytes. DNA quality was checked by β-globin PCR to determine the presence of PCR-amplifiable DNA. The PCR products were separated on 1.5% agarose gels. DNA marker, low-mass DNA molecular weight marker VIII (Roche; fragment lengths in base pairs are indicated); G, granulocyte; L, lymphocyte; M, monocyte; N, negative (H_2O) control; +, MCPyV-positive MCC control.

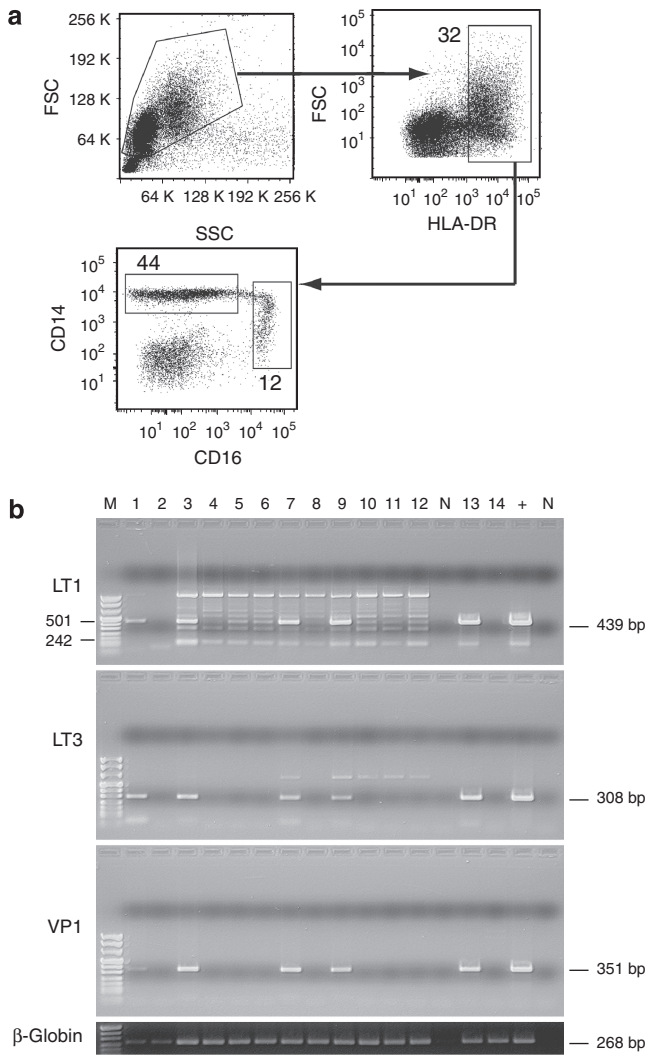

Figure 2. Detection of MCPyV DNA in purified leukocyte subsets. (**a**) Isolation of HLA-DR$^+$CD14$^+$CD16$^-$ inflammatory monocytes and HLA-DR$^+$CD14^{lo}CD16$^+$ resident monocytes from peripheral blood mononuclear cells (PBMCs) using FACS sorting. The HLA-DR$^+$ PBMCs were determined, and the two monocyte populations (CD14$^+$CD16$^-$ and CD14^{lo}CD16$^+$) were isolated. Percentages of fractionated cell populations are given. (**b**) Detection of Merkel cell polyomavirus (MCPyV) DNA in patient 1. Genomic DNA from cell-free serum, whole blood, isolated blood cells, and various skin tumors of patient 1 was amplified with primers specific for the LT1, LT3, or VP1 genes of MCPyV. The specific LT1 amplification product is 439 bp in size. The prominent band at about 1,000 bp observed with the LT1 primer pair corresponds to an unspecific amplification product due to cross-reactivity with a sequence on human chromosome 6. DNA isolated from: 1, cell-free serum; 3, PBMCs; 5, lymphocytes; 7, monocytes; 9, CD14$^+$CD16$^-$ monocytes; 11, CD14^{lo}CD16$^+$ monocytes; 13, urine of patient 1. DNA isolated from: 2, cell-free serum; 4, PBMCs; 6, lymphocytes; 8, monocytes; 10, CD14$^+$CD16$^-$ monocytes; 12, CD14^{lo}CD16$^+$ monocytes; 14, urine of control individual. M, low-mass DNA molecular weight marker VIII (Roche; fragment lengths in base pairs are indicated); N, normal skin; +, MCC-positive control.

marginated pool of monocytes (Geissmann *et al.*, 2008). CD14$^+$CD16$^-$ ''inflammatory'' monocytes, on the other hand, have the capacity to home to inflamed tissues in response to the chemokine (CC motif) ligand 2/monocyte chemotactic protein-1. This dichotomy in monocyte phenotype and function prompted us to analyze resident and inflammatory monocytes from the peripheral blood of patient 1 separately (Figure 2a). When we tested both, i.e., FACS-sorted isolated HLA-DR$^+$CD14$^+$CD16$^-$ inflammatory monocytes and HLA-DR$^+$CD14^{lo}CD16$^+$ resident monocytes for MCPyV DNA, we found MCPyV to be selectively associated with the inflammatory monocyte population (Figure 2b). Again, sequencing confirmed the identity of the MCPyV PCR products. Therefore, we conclude that inflammatory monocytes might have the unique capacity to take up MCPyV, and carry it throughout the body.

Recruitment of inflammatory monocytes to inflamed skin lesions in psoriasis patients

HLA-DR$^+$CD14$^+$CD16$^-$ inflammatory monocytes are specifically recruited to sites of inflammation through the chemokine receptor, chemokine (CC motif) receptor 2. We wanted to know whether a similar mechanism operates in psoriasis. First, we carefully analyzed defined PBMC in both normal individuals and psoriasis patients. Specifically, we determined the amount of resident and inflammatory

monocytes in the blood of healthy individuals and of psoriasis patients. For FACS analysis, PBMCs of three healthy individuals and five psoriasis patients were used. The psoriasis patients had significantly more inflammatory monocytes in the blood when compared with normal healthy controls ($P=0.04$, Mann–Whitney test; Supplementary Figure S1 online).

Next, we asked whether monocytes might get recruited to psoriatic skin lesions through chemokine (CC motif) receptor 2. Indeed, we observed elevated expression of the chemokine (CC motif) receptor 2 ligand monocyte chemotactic protein-1/chemokine (CC motif) ligand 2 in psoriatic skin, suggesting that inflammatory monocytes may use this chemoattractant pathway for recruitment (Ginhoux *et al.*, 2006; Supplementary Figure S2 online). Inflammatory monocytes can differentiate to macrophages at sites of inflammation (Ginhoux *et al.*, 2006). Therefore, we analyzed the inflammatory skin infiltrate in psoriasis patients by immunohistochemistry using CD68 for macrophages. In psoriatic skin, we observed significantly more macrophages when compared with normal skin samples (mean ± standard deviation, 12.48 ± 3.75 CD68-positive cells per high-power field in psoriatic skin vs 4.29 ± 1.95 CD68-positive cells per high-power field; $P=0.0007$, Mann–Whitney test; Supplementary Figure S2 online).

Taken together, this is correlative evidence for the fact that inflammatory monocytes may reach psoriatic lesions through chemokine (CC motif) receptor 2 and differentiate to macrophages *in situ*. During this process, MCPyV-positive inflammatory monocytes may import the virus into the inflamed skin lesions.

Detection of MCPyV DNA in the urine of both patients

Other members of the polyomavirus family, e.g., BKV and JCV, can persist in the urinary tract and can be transmitted through the urine (Markowitz *et al.*, 1993). This prompted us to test the urine of both described patients, and we found it to be positive for MCPyV DNA (Figure 2b). MCPyV was not detected in the urine of normal healthy individuals ($n=7$, of which five were females, median age 43, range 25–60) and of psoriasis patients without MCC ($n=6$, of which four were females, median age 51, range 35–81). So far, it remains unclear whether MCPyV is able to persist in the urinary tract or whether the urinary route represents a route of virus excretion. In addition, we cannot be sure that this virus is still infectious or whether the PCR signal corresponds to viral particles that have been neutralized by antibodies or to viral fragments.

DISCUSSION

This study identifies monocytes, and more specifically, $CD14^+CD16^-$ inflammatory monocytes, as a reservoir of MCPyV. In addition, we detected MCPyV in the urine. Although our observation relates to isolated cases, these findings give important hints about the tropism and mode of transmission of MCPyV.

Polyomaviruses are widespread among humans: about 80% of all adults have antibodies to BKV, JCV, or MCPyV (Padgett and Walker, 1973; Egli *et al.*, 2009). It is assumed that these viruses persist lifelong and get re-activated upon immune alterations. BKV, JCV, and MCPyV have been associated with tumors: BKV has been found in brain tumors and insulinomas, and JCV seems to be associated with human tumors of the central nervous system (Dorries, 1998). Very recently, MCPyV has been implicated in the etiology of MCC, a malignancy of neuroendocrine cells of the skin (Feng *et al.*, 2008). As the reactivation of polyomaviruses may have detrimental consequences, particularly under regimens of therapeutic immunomodulation, it is important to understand the life cycle of these pathogens. It is agreed upon that the main site of BKV and JCV persistence in healthy individuals is the kidney (Dorries, 1998). PBMCs have been proposed as another site of persistence, at least in immunocompromised hosts (Azzi *et al.*, 1996). For healthy individuals, however, it is less clear whether BKV and JCV can persist in PBMCs. Depending on the study, BKV and JCV sequences were found in PBMCs of 0–90% of healthy individuals tested. This huge variation may rather point toward events of recent infection or viral reactivation in a subgroup of patients and not toward persistence (Dolei *et al.*, 2000).

In contrast to other polyomaviruses, the site of persistence and the mode of transmission have not yet been addressed for MCPyV. Two recent studies did not detect MCPyV in a cohort of 40 or 45 healthy blood donors (Kassem *et al.*, 2008; Duncavage *et al.*, 2009). Although this shows that MCPyV does not seem to persist frequently in PBMCs, the infectious history of MCPyV in this patient cohort was not taken into account. Only 25% of healthy individuals are seropositive for MCPyV350 (Kean *et al.*, 2009), and it remains to be observed whether viral persistence in blood is equally low in seropositive versus seronegative patients.

In this study we described two patients with a known infectious history of MCPyV-positive skin lesions. Both patients harbored MCPyV in skin lesions at various anatomic locations, associated with mixed inflammatory infiltrates. This led us to identify a hematologic reservoir, more specifically $CD14^+CD16^-$ inflammatory monocytes. These cells may take up and digest opsonized, i.e., neutralized virus. Alternatively, inflammatory monocytes may exert an effect as efficient pathogen shuttles throughout the body (Drevets *et al.*, 2004). These cells are able to enter inflammatory sites and might act as ''Trojan horses'' releasing their pathogenic load *in situ* (Supplementary Figure S3 online). One of our patients suffered from psoriasis well before developing MCC. Psoriasis lesions express chemokine (CC motif) ligand 2/monocyte chemotactic protein-1, a chemokine known to selectively attract inflammatory monocytes (Deleuran *et al.*, 1996). Infected monocytes localizing within lesions of inflammatory skin diseases, such as psoriasis, may release MCPyV locally, infect Merkel cells, and may induce MCC (Wollina and Mahrle, 1992). In addition, for the immunosuppressed kidney transplant recipient, we were able to show the presence of MCPyV in blood monocytes. This patient suffered from multiple benign and malignant skin neoplasms accompanied by chronic inflammatory infiltrates. Melanoma (Graves *et al.*, 1992), squamous cell carcinoma (Koide *et al.*, 2004), basal cell

carcinoma (Welss *et al.*, 2003), HPV-16-infected tissue (Riethdorf *et al.*, 1996), and potentially other skin neoplasms also express monocyte chemotactic protein-1/chemokine (CC motif) ligand 2, and hence the mechanism of MCPyV entry to the skin may be similar in both patients.

The scenario of inflammatory monocytes carrying MCPyV away from MCCs into the circulation and to other sites of the body is less likely. Inflammatory monocytes usually extravasate to inflamed skin in which they differentiate to macrophages and Langerhans cells (Ginhoux *et al.*, 2006). The local inflammatory milieu induces maturation of Langerhans cells and migration to the closest draining lymph node (Kissenpfennig *et al.*, 2005). In other words, inflammatory monocytes have not been described to re-enter the circulation from inflammatory sites. Therefore, it is likely that circulating monocytes have acquired MCPyV outside inflammatory lesions or MCCs.

In both of our patients, we detected MCPyV DNA in cell-free serum. The MCPyV DNA signal in serum may relate to free DNA, MCPyV particles coupled to neutralizing antibodies, or infectious virus. In the absence of a plaque-forming *in vitro* assay to quantify infectious MCPyV, we could not distinguish between these three possibilities. However, the presence of MCPyV DNA in the urine in both patients is compatible with the idea that MCPyV gets neutralized in the bloodstream and is then excreted through the kidneys. Alternatively, the kidney may potentially provide a site of MCPyV persistence, as for other polyomaviruses.

Although our observations relate to isolated cases, these findings raise important issues related to the mode of transmission of MCPyV. As long as it is not fully clarified whether monocytes constitute a previously unknown long-term reservoir of MCPyV or a shuttle for virus transmission, caution must be exerted when transplanting organs, or when transfusing blood from MCPyV-positive donors.

MATERIALS AND METHODS

Patients

This study was approved by the ethics review board of the Canton Zurich, Switzerland. Written informed consent was obtained from all patients. The study was conducted with strict adherence to the Declaration of Helsinki Principles.

The medical records of the patients were reviewed and the relevant information was extracted. Formalin-fixed, paraffin-embedded skin biopsies were reviewed by a board-certified pathologist (MP) and a board-certified dermatopathologist (WK). Control lesions of immunocompetent patients were randomly selected from our archive. These patients had never been treated with immunosuppressive drugs nor did they suffer from immunodeficiencies.

DNA extraction

A total of three consecutive 10 μm paraffin sections from each tissue sample were used for DNA extraction. After deparaffinization, all tissues were subjected to proteinase K digestion in lysis buffer (50 mM Tris–HCl, pH 8.5, 1 mM EDTA, 0.5% Tween-20) at 53 °C overnight. DNA was purified using the QIAamp DNA mini kit (Qiagen, Hilden, Germany) and was directly used for PCR.

We collected blood and urine from patients 1 and 2 as well as from age- and sex-matched healthy control individuals. DNA from whole blood, PBMCs, and sorted peripheral blood cell fractions was extracted using the QIAamp DNA blood mini kit (Qiagen). DNA from cell-free serum and urine was extracted using the QIAamp viral RNA kit (Qiagen).

MCPyV detection by PCR

DNA quality was confirmed using β-globin PCR (GH20 primer 5′-GAAGAGCCAAGGACAGGTAC-3′; PCO4 primer 5′-CAACTT CATCCACGTTCACC-3′). For MCPyV DNA detection we used the primer sets LT1, LT3, and VP1 as described (Feng *et al.*, 2008). PCR was performed with genomic DNA using 40 cycles for each primer set with the FastStart Taq polymerase (Roche, Basel, Switzerland) in a final volume of 50 μl. Genomic DNA from a MCPyV-positive and sequence-confirmed MCC was used as a positive control; water and normal skin instead of DNA were used as negative controls. The PCR products were detected using ethidium bromide staining after electrophoretic migration through 1.5% agarose gels.

Sequence analyses

Amplified MCPyV PCR products were purified before sequencing using the High Pure PCR product purification kit (Roche). PCR products were submitted for automated sequencing using a Roche FLX genome sequencer (Microsynth, Balgach, Switzerland). The resulting DNA sequences were aligned against the reference sequences of the National Center for Biotechnology Information Entrez Nucleotide database using the center's Blast program (gb|EU375803.1 Merkel cell polyomavirus isolate MCC350; gb|EU375804.1 Merkel cell polyomavirus isolate MCC339).

Isolation of PBMCs

Peripheral blood mononuclear cells were isolated from whole-blood samples using Ficoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation as described (Dolei *et al.*, 2000).

FACS sorting of PBMCs

Peripheral blood mononuclear cells were stained with Pacific blue-conjugated anti-HLA-DR, phycoerythrin-conjugated anti-CD16, and FITC-conjugated anti-CD14 (BD Pharmingen, San Diego, CA). The two major human monocyte subsets, $CD14^{+}CD16^{-}$ and $CD14^{lo}CD16^{+}$, were sorted on a BD FACS Aria (BD Pharmingen) and analyzed using FlowJo v.7 software (Tree Star, Mountain View, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We are very grateful to Dr Carmen Laetsch, board-certified dermatologist, Bad Ragaz, Switzerland, for providing clinical data, and to Vinko Tosevski for expert cell sorting.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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