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Spontaneous Regression of Highly Immunogenic *Molluscum contagiosum* Virus (MCV)-Induced Skin Lesions Is Associated with Plasmacytoid Dendritic Cells and IFN-DC Infiltration

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Molluscum contagiosum virus (MCV) infection induces self-limiting cutaneous lesions in an immunocompetent host that can undergo spontaneous regression preceded by local inflammation. On histology, a large majority of MCV-induced lesions are characterized by islands of hyperplastic epithelium containing infected keratinocytes and surrounded by scarce inflammatory infiltrate. However, spontaneous regression has been associated with the occurrence of a dense inflammatory reaction. By histology and immunohistochemistry, we identified MCV-induced lesions showing a dense inflammatory infiltrate associated with cell death in keratinocytes (inflammatory *Molluscum contagiosum* (I-MC)). In I-MC, hyperplastic keratinocytes were highly immunogenic as demonstrated by the expression of major histocompatibility complex class I and II molecules. Immune cell infiltration consisted of numerous cytotoxic T cells admixed with natural killer cells and plasmacytoid dendritic cells (PDCs). Accordingly, a type I IFN signature associated with PDC infiltration was demonstrated in both keratinocytes and inflammatory cells. Among the latter, a cell population resembling IFN-DC ($CD123^+CD11c^+CD16^+CD14^+MxA^+$) was identified in proximity to islands of apoptotic keratinocytes. *In vitro*–generated IFN-DCs expressed a strong cytotoxic signature, as demonstrated by high levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL). This study establishes a previously unreported model to underpin the role of innate immune cells in viral immune surveillance.

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INTRODUCTION

Molluscum contagiosum (MC) is a common benign viral infection affecting the skin and mucosal membranes. The *Molluscum contagiosum* virus (MCV), a DNA poxvirus, is responsible for the disease. In immune-competent hosts, MCV-induced lesions can undergo spontaneous regression. In immunodeficient patients MCV is responsible for more extensive infections (Cotton *et al.*, 1987; Schwartz and Myskowski, 1992). Many patients and dermatologists note that manipulation of a lesion, which leads to the extrusion of molluscum bodies into the dermis and their exposure to immune cells, can lead to marked perilesional erythema, followed by the regression of other, non-traumatized lesions (Epstein, 1992; Brown *et al.*, 2006). These data indicate that the immune system has a critical role in the control of viral spread.

A large majority of MCV-induced tumor-like lesions are characterized by islands of hyperplastic epithelium containing infected keratinocytes and surrounded by scarce inflammatory infiltrates. The latter finding suggests that the virus adopts escape mechanisms from local immune surveillance (reviewed in Moss *et al.*, 2000; Seet *et al.*, 2003). Genes encoded by poxviruses can target key host immune genes such as major histocompatibility complex (MHC) class I molecules, chemokines, IFNs, and ILs, which ultimately reduce the immunogenicity of infected cells and hamper the recruitment and function of immune cells to the primary site of infection (Senkevich *et al.*, 1996; Krathwohl *et al.*, 1997;

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Abbreviations: GrB, granzyme B; IFN-DC, IFN-induced dendritic cell; I-MC, inflammatory Molluscum contagiosum*; MCV,* Molluscum contagiosum *virus; MHC, major histocompatibility complex; NI-MC, non-inflamed* Molluscum contagiosum*; PDC, plasmacytoid dendritic cell/IFN-producing cell; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand*

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Damon *et al.*, 1998; Luttichau *et al.*, 2000). Notably, in a subgroup of MCV-induced lesions a dense dermal infiltrate composed by macrophages and numerous T cells comprising a portion of CD30+ activated T cells is observed (Guitart and Hurt, 1999). Remarkably, dense local inflammation has been associated with spontaneous regression, as demonstrated by follow-up biopsies obtained from occasional case reports (Steffen and Markman, 1980; Epstein, 1992).

This set of evidences suggests that in an immune-competent host the occurrence of a spontaneous rejection is tightly associated with a local immune response. To our knowledge this represents a unique model to trace cellular events leading to immune-mediate antiviral response to cutaneous poxviruses. During a screening of MCV-induced skin lesions, we identified cases showing a strong inflammatory reaction associated with histological evidence of keratinocyte cell death. We surmise that this finding was representative of an ongoing and traceable active regression induced by infiltrating immune cells. By extending this analysis, we found that the occurrence of histological regression of MCV lesions was strikingly associated with local inflammation and increased immunogenicity of keratinocytes. Immune cell infiltration in regressing MC consists of cytotoxic T cells and innate immune cells. Among the latter, clusters of type I IFN-producing plasmacytoid dendritic cells represented a common feature. Of note, among type I IFN-targeted cells, numerous IFN-induced dendritic cells (IFN-DCs) formed clusters around areas of hyperplastic epithelium. IFN-DCs, when generated *in vitro*, are equipped with a strong cytotoxic signature, as demonstrated by high levels of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), and granulolysin.

RESULTS

A subgroup of MCV-induced skin lesions is heavily infiltrated by innate and adaptative immune cells

Scant infiltration of leukocytes is typically observed in MCV-induced skin lesions (Heng *et al.*, 1989). Based on their histology and content of $CD45RB^+$ leukocytes, 36 MCV-induced lesions were categorized in two main patterns. The first pattern (10 cases) was represented by non-inflamed lesions (NI-MC) infiltrated by very scant $CD45^+$ leukocytes (Figure 1a), whereas the second pattern (26 cases; from here on referred to as inflammatory MC (I-MC)) showed consistent amounts of $CD45^+$ infiltrating cells (Figure 1b). Inflammatory cells in I-MC were either diffuse in the dermis or surrounded islands of hyperplastic epithelium; of note, intraepithelial infiltration was normally limited to the dermoepidermal boundary (Figure 1b). In NI-MC the inflammatory cell population was represented by rare $CD163^+$ macrophages and scattered $CD11c^+$ cells (not shown). In contrast, I-MC biopsies contained numerous $CD3^+$ T cells (Figure 1c) admixed with rare $CD20^+$ B cells and $CD3^-Perforin^+$ $CD56^+$ natural killer cells (not shown). Among lymphoid cells only T cells directly surrounded the infected epithelium. The antiviral response to Poxviruses requires CD8 T cells sustained by T-helper 1 (Th1) cytokines (Stanford and McFadden, 2005). $CD3^+$ T cells mainly consisted of $CD8^+$ cytotoxic T cells expressing granzyme B (GrB) and perforin, as revealed by double staining with immunohistochemistry (Figure 1d); notably, a fraction of them also coexpressed the activation marker CD30 (Figure 1e). The remaining T-cell population included $CD4^+$ T cells. Based on immunostaining for T-bet and Foxp3 (forkhead box P3), a Th1-type polarization of the immune response was obvious in I-MC, as revealed by predominant nuclear expression of T-bet in lymphoid cells (Figure 1f).

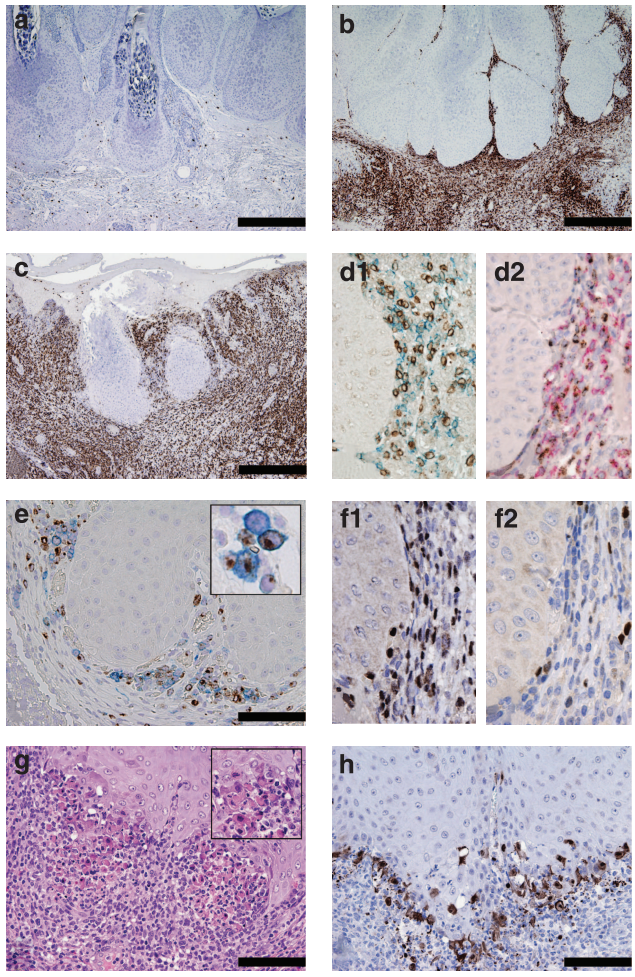

Figure 1. Cytotoxic T-cell infiltration and apoptotic cell death characterize inflammatory *Molluscum contagiosum* (I-MC). Sections from non-inflamed *Molluscum contagiosum* (NI-MC; **a**) and I-MC (**b**–**h**) are immunostained for CD45RB (**a**, **b**), CD3 (**c**), CD8 (**d1** and **d2**), CD30 (**e**), perforin (**d1**), granzyme B (GrB; **d2** and **e**), T-bet (**f1**), Foxp3 (**f2**), hematoxylin/eosin (**g**), and cleaved caspase 3 (**h**). In NI-MC, scattered $CD45^+$ cells are observed (**a**), whereas I-MC are densely infiltrated by $CD45^+$ leukocytes (**b**); intraepithelial inflammatory cells are very rare. I-MC contain numerous $CD3^+$ T cells (**c**), including $CD8^+$perforin$^+$ (**d1**), $CD8^+GrB^+$(**d2**), and $CD30^+$ GrB^+ cells (**e**, inset). The large majority of T cells express T-bet (**f1**), with only rare $Foxp3^+$ cells (**f2**). In I-MC, numerous apoptotic keratinocytes occur at the boundary between epithelium and stroma (**g**, inset, **h**). Secondary antibodies are revealed with diaminobenzidine (DAB; brown in **a**–**f** and **h**), new fucsin (red, CD8 in **d2**), and Ferangi blue (blue, CD8 in **d1** and CD30 in **e**). Original magnification ×40 (**a**–**c**; scale bar = 500 μm), ×200 (**e**, **g**, and **h**; scale bar = 100 μm), and ×400 (**d1**, **d2**, **f1**, **f2**, and insets in **e** and **g**; scale bar = 50 μm).

Increased immunogenicity and apoptotic cell death of infected keratinocytes in I-MC

On hematoxylin and eosin stain, we noticed that apoptotic cell death represented a common feature of I-MC (24/26 cases; 92.3%) and this observation was supported by the demonstration of active caspase 3 expression by sparse keratinocytes (Figure 1g and h) at the periphery of infected epithelial lobules. No reactivity for anti-active caspase 3 was observed in all 10 NI-MC cases (Supplementary Figure S1 online). The restriction of the foci of cell death to I-MC along with their distribution at the periphery of MCV epithelium suggested an ongoing immune response against virus-infected keratinocytes. MCV-encoded genes can interfere with the level of immunogenicity of infected cells by targeting MHC class I (Senkevich *et al.*, 1996; Senkevich and Moss, 1998). Tissue sections from 10 I-MC and 10 NI-MC cases were stained for MHC class I (MHC-I) and II molecules (MHC-II), transporter associated with antigen processing 1 (TAP1) and 2 (TAP2), Tapasin, and NF-κB (Figure 2a–h). In NI-MC cases (10/10 cases), the large majority of epithelial cells lacked MHC-I, MHC-II and TAP1 and TAP2, whereas NF-κB was mostly retained in the cytoplasm. Remarkably, I-MC showed marked changes of MHC molecules, with upregulation of MHC-I, MHC-II, TAP1, and TAP2 (10/10 cases) and nuclear translocation of NF-κB (7/10 cases); all these alterations were particularly evident in keratinocytes at the periphery of infected epithelium.

In summary, a bimodal spectrum of presentation is observed in MCV-induced skin lesions. From one end, NI-MC completely lack histological evidence of immune-mediated regression and loose expression of MHC-I and II molecules. On the other end, I-MC lesions are highly immunogenic lesions with histological feature of an ongoing immune-mediated regression process.

Infiltration of type I IFN-producing PDCs is a common feature of I-MC

Plasmacytoid dendritic cells/IFN-producing cells (PDCs) represent a well-characterized cell population (Facchetti *et al.*, 2003; Colonna *et al.*, 2004; Liu, 2005) capable of producing large amounts of type I IFN with a potential role in antiviral defense mechanisms. Although PDCs were completely absent in NI-MC (10/10 cases; not shown), they were regularly found in all 26 cases of I-MC (8/26 score 1; 18/26 score 2). PDCs were identified as medium-sized round cells positive for CD123, TCL1 (T cell leukemia-1; Vermi *et al.*, 2004; Santoro *et al.*, 2005) CD303/BDCA2 (blood dendritic cell antigen-2), CD2AP (CD2-associated protein), and BCL11A (B-cell lymphoma/leukemia 11A). PDCs were distributed either in the form of variable-sized clusters within dermal inflammatory nodules or as rim surrounding islands of infected epithelium (Figure 3a and b). Using double immunohistochemistry we found that BDCA2$^+$ PDCs showed cytoplasmic expression of GrB (Figure 3c), as previously reported (Vermi *et al.*, 2004, 2009; Santoro *et al.*, 2005); however, when measured, the GrB content was reduced or absent in 48.4% of PDCs (Figure 3d). Activation of PDCs by viruses results in the production of high levels of type I IFN via Toll-like receptor-dependent signals. Expression of MxA has been used as a surrogate marker of type I IFN production (Farkas *et al.*, 2001; Santoro *et al.*, 2005; Vermi *et al.*, 2003). Immunostaining for anti-MxA showed clearcut differences between the two subgroups of MC lesions. In I-MC, a diffuse and strong cytoplasmic expression of the protein was detectable in keratinocytes and inflammatory cells (20/20 cases; Figure 3e), whereas NI-MC were either negative (7/10 cases) or showed focal and weaker MxA reactivity (3/10 cases; Figure 3f). To further investigate the molecular effects of type I IFN production, the tyrosine-phosphorylated form of STAT1 (signal transducer and acti-

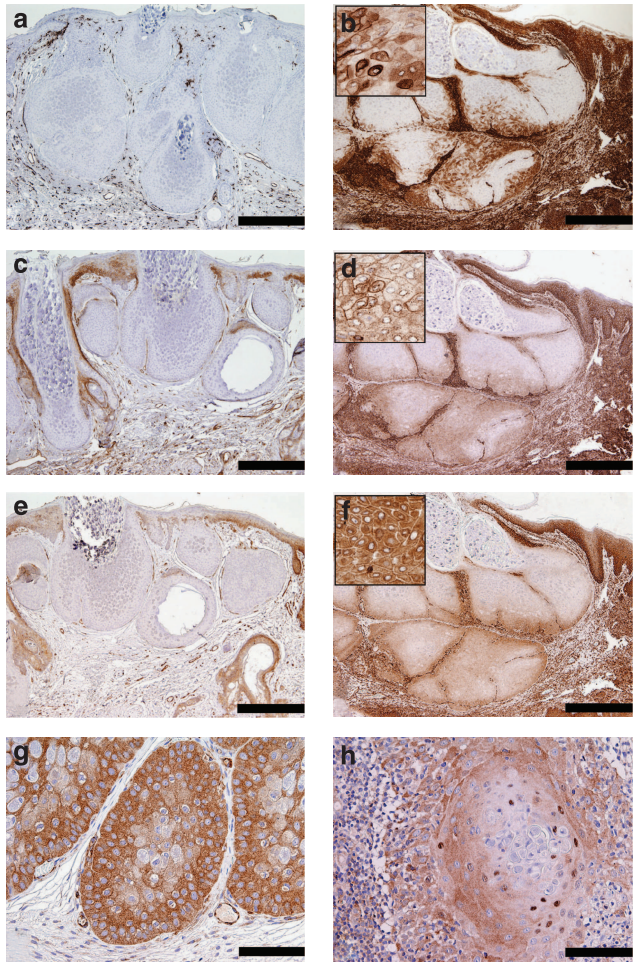

Figure 2. Differential expression of major histocompatibility complex (MHC)-I, MHC-II, TAP1, and NF-κB in *Molluscum contagiosum* (MC). Sections from non-inflamed MC (NI-MC; **a**, **c**, **e,** and **g**) and inflammatory MC (I-MC; **b**, **d**, **f,** and **h**) are immunostained for major histocompatibility complex-11 (MHC-II; **a**, **b**), MHC-I (**c**, **d**), TAP1 (**e**, **f**), and NF-κB (**g**, **h**). In NI-MC, MHC-II (**a**), MHC-I (**c**), and TAP1 (**e**) are restricted to stromal cells and, for MHC-I and TAP1, to areas of normal skin. I-MC shows strong reactivity for all these molecules (**b**, **d,** and **f** with corresponding insets), at the periphery of infected epithelium. In NI-MC, NF-κB is restricted to the cytoplasm (**g**), whereas the same molecule localizes to the nucleus of keratinocytes in I-MC (**h**). Secondary antibodies revealed with diaminobenzidine (DAB). Original magnification × 40 (**a**–**f**; scale bar = 500 μm), × 200 (**g** and **h**; scale bar = 100 μm), and × 400 (insets in **b**, **d,** and **f**; scale bar = 50 μm).

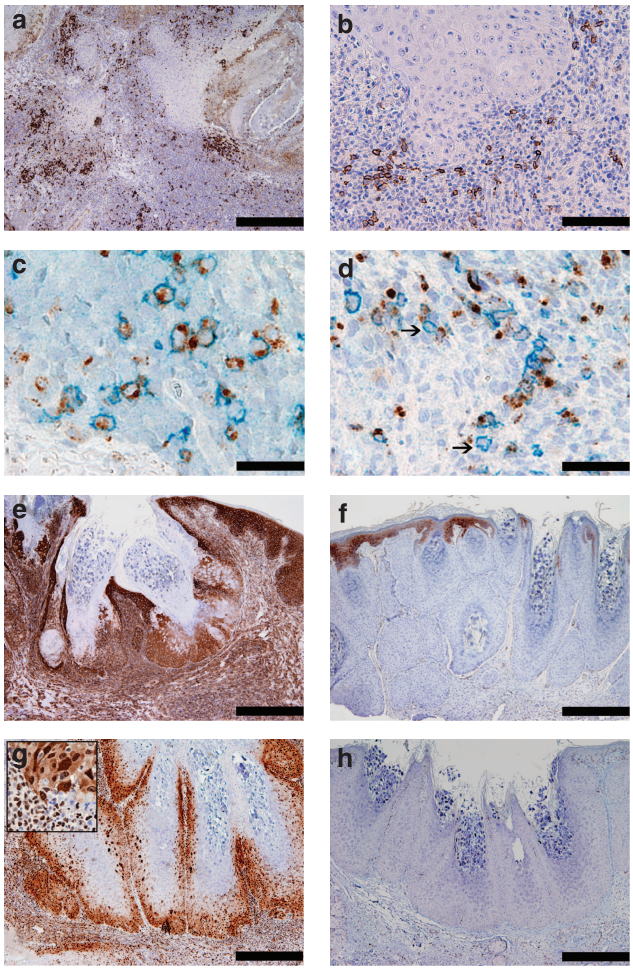

Figure 3. Accumulation of plasmacytoid dendritic cells (PDCs) in inflammatory *Molluscum contagiosum* (I-MC) is associated with type I IFN protein expression signature. Sections from I-MC (**a**–**e**, **g**) and non-inflamed (NI)-MC (**f** and **h**) are immunostained for CD123 (**a**), BDCA2 (**b**–**d**), granzyme B (GrB; **c** and **d**), MxA (**e** and **f**), and STAT1pY701 (**g** and **h**). In I-MC, PDCs occur in the deep dermis or surround islands of infected epithelium (**a**, **b**) and may contain large GrB$^+$ granules (**c**), or totally lack GrB reactivity (**d**, black arrows). In I-MC, a diffuse and strong reactivity for MxA (**e**) and nuclear STAT1pY701 (**g**) of keratinocytes and inflammatory cells is obvious (inset in **g**). In NI-MC, reactivity for MxA and STAT1pY701 is weak and focal (**f** and **h**). Secondary antibodies revealed with diaminobenzidine (DAB; brown in **a**–**h**) and Ferangi blue (blue, BDCA2 in **c** and **d**). Original magnification ×40 (**a**, **e**–**h**; scale bar = 500 μm), ×100 (**a**; scale bar = 200 μm), ×200 (**b**; scale bar = 100 μm), ×400 (inset in **g**; scale bar = 50 μm), and ×600 (**c** and **d**; scale bar = 20 μm).

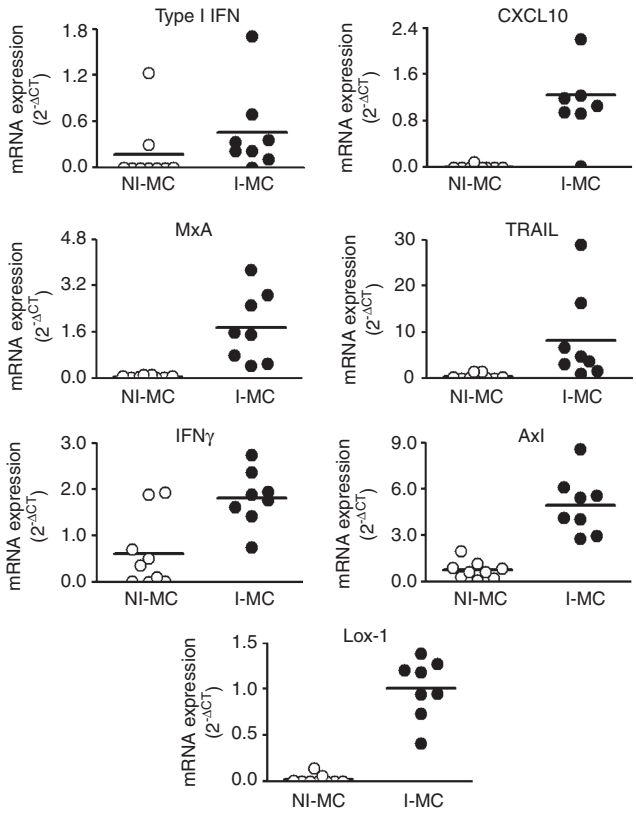

Figure 4. Expression of IFNs and IFN-inducible genes in *Molluscum contagiosum* (MC). Gene expression was evaluated using mRNA extracted from inflammatory MC (I-MC) and non-inflamed MC (NI-MC). mRNA expression ($2^{-\Delta CT}$) was normalized to 18S rRNA. Results are expressed as arbitrary units.

vator of transcription 1) was tested by immunohistochemistry. In response to type I IFN binding, the JAK–STAT pathway is activated and the STAT1 subunit becomes phosphorylated. Nuclear translocation of a trimeric complex including phosphorylated STAT1 binds IFN-regulated response elements and modulates the transcription of IFN-stimulated genes (Borden *et al.*, 2007). We found a strong nuclear reactivity for anti-STAT1pY701 in I-MC (10/10 cases; Figure 3g). In particular, STAT1pY701$^+$ cells were represented by numerous keratinocytes surrounding infected cells as well as by leukocytes infiltrating the dermis (inset in Figure 3g); only very weak and focal reactivity was observed in keratinocytes of NI-MC (Figure 3h). Finally, to validate our hypothesis that type I IFNs are locally produced in I-MC, RNA was extracted from 17 MCV-induced lesions. Compared with NI-MC, IFN-α, IFN-γ, MxA, and CXCL10 (chemokine (C-X-C motif) ligand 10) were strongly upregulated in I-MC containing PDCs and IFN-DCs (Figure 4).

Altogether, these data indicate that large numbers of type I IFN-producing PDCs infiltrate I-MC, and IFN target cells are represented by keratinocytes and surrounding immune cells.

A previously unreported population of CD11c$^+$CD123$^+$ DCs resembling the so-called "IFN-DC" infiltrate I-MC

In the large majority of I-MC cases (25/26; 96%), we noticed a population of large CD123$^+$ cells showing abundant cytoplasm and an elongated, slightly irregular nucleus (Figure 5a and c). These cells were frequently found in close proximity of infected epithelium, admixed with PDCs. They were negative for other PDC markers (BDCA2 and CD2AP; not shown), CD207, and CD1a (not shown), as well as for CD3, CD20, and CD56, but strongly reacted with CD11c (Figure 5b and d). Notably, a large majority of them were also negative for the macrophage-specific marker CD163

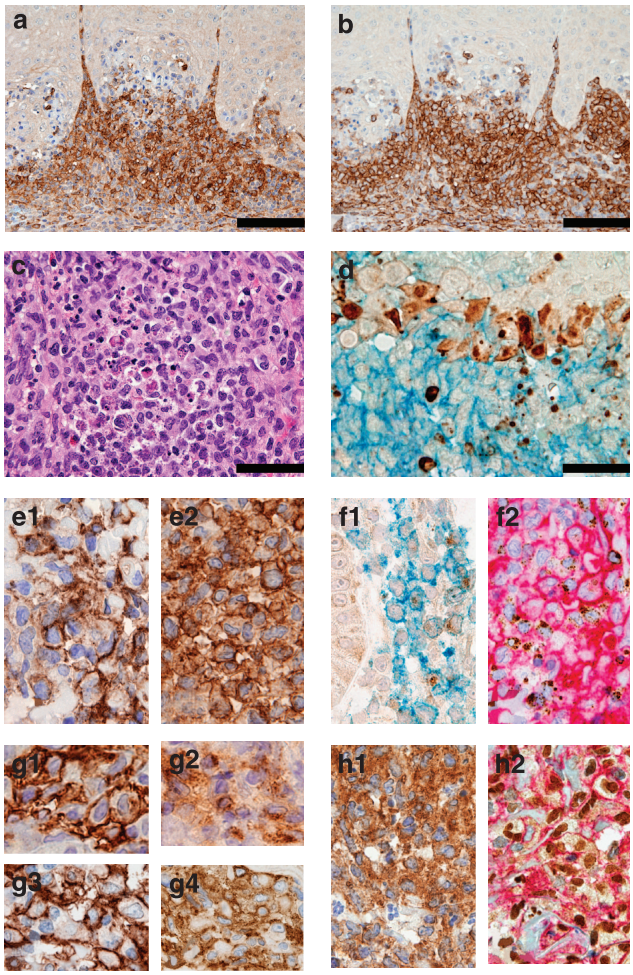

Figure 5. IFN-induced dendritic cells (IFN-DCs) occur in inflammatory *Molluscum contagiosum* (I-MC) lesions. All sections are from I-MC and stained for CD123 (**a**), CD11c (**b**, **d**, **f,** and **h2**), hematoxylin/eosin (**c**), active caspase-3 (**d**), CD14 (**e1**), CD16 (**e2**), TRAIL (**f1**), GrB (**f2**), CCR7 (**g1**), CD83 (**g2**), CD40 (**g3**), MHC-II (**g4**), MxA (**h1**), and STAT1pY701 (**h2**). Serial sections (**a, b**) illustrate numerous large CD123$^+$ cells (**a**) coexpressing CD11c (**b**) with round-to-elongated nucleus and ample cytoplasm (**c**), localized in areas of keratinocytes apoptosis (**a**–**d** and **f**). These cells express CD14 (**e1**), CD16 (**e2**), CCR7 (**g1**), CD40 (**g3**), MHC-II (**g4**), MxA (**h1**), and STAT1pY701 (**h2**). A more limited fraction is also positive for TRAIL (**f1**) GrB (**f2**) and CD83 (**g2**). Secondary antibodies revealed with diaminobenzidine (DAB; brown in **a**, **b**, **d**–**h2**), new fucsin (red, CD11c in **f2** and **h2**), and Ferangi blue (blue, CD11c in **d** and **f1**). Original magnification ×200 (**a**, **b,** and **f**; scale bar = 100 μm), ×400 (**c**–**e**, **g**, **h**; scale bar = 50 μm).

(not shown). The puzzling phenotype and morphology of these cells prompted us to further characterize them by using single immunostains on serial sections and, when feasible, double immunostains. Among the tested markers, we found that a large majority of CD123$^+$CD11c$^+$ cells were also CD14^{dim} and strongly reacted for CD16, CD40, HLA-DR, MxA, and STAT1pY701 (Figure 5e, g3, g4, and h). Remarkably, a fraction of them also stained for CCR7 (chemokine (C-C motif) receptor 7), CD83 (Figure 5g1 and g2), and CD208/DC-LAMP (not shown). We excluded a myeloid origin of these cells either by myeloperoxidase immunostain (that reacted with only few of them) and the chloroacetate esterase histochemistry, which was negative (data not shown).

Overall, the phenotype of these cells is reminiscent of the so-called ''IFN-DC,'' a dendritic cell type obtained *in vitro* by short exposure to GM-CSF and type I IFN (Santini *et al.*, 2000). In addition to the ability of IFN-DCs to cross-present viral antigen, they can exert TRAIL- and GrB-dependent effector function. We found that CD11c$^+$CD123$^+$ cells in I-MC were remarkably located in areas of keratinocyte cell death and a fraction of them expressed TRAIL, GrB (Figure 5f), but not perforin (not shown).

In summary, these data indicate that a, to our knowledge previously unreported, cell type with unprecedented morphological and phenotypical feature and resembling the so-called ''IFN-DC'' is part of the local immune reaction in I-MC.

In vitro conditioning of peripheral blood monocytes by type I IFN generates CD11c$^+$CD123$^+$ IFN-DCs with a strong cytotoxic signature

To further extend the comparison between the CD123$^+$CD11c$^+$ population detected *in vivo* and ''IFN-DC,'' circulating monocytes were cultured *in vitro* in the presence of GM-CSF and type I IFN. At the end of the 4-day culture protocol, a certain degree of heterogeneity was present in the cell culture, with a first population characterized by low expression of CD1a and CD14 and high expression of MxA and CD16 (Figure 6a), and a second population higher positive for CD1a but poorly expressing MxA and CD16. In their whole, IFN-DCs were CD123$^+$ and CD11c$^+$ (Figure 6b) and expressed higher levels of FasL and TRAIL mRNA than classic monocyte-derived DCs (Figure 6b). Therefore, these *in vitro* data further strengthen the correlation between tissue CD123$^+$CD11c$^+$ cells and IFN-DCs. Finally, using quantitative PCR (qPCR) of NI-MC versus I-MC we found that Axl, TRAIL, and Lox1, the genes highly expressed in IFN-DCs (Scutera *et al.*, 2009; Parlato *et al.*, 2010), are strongly upregulated in I-MC (Figure 4).

DISCUSSION

In immune-competent patients, spontaneous regression of MCV-induced lesions is commonly preceded by clinical signs of inflammation (Epstein, 1992; Brown *et al.*, 2006). The cellular mechanisms underling this event are still incompletely understood. This study documents the occurrence of a composite inflammatory reaction to MCV-induced lesions associated with histological regression. We found that histological features of ongoing regression were tightly correlated with the occurrence of a composite local immune reaction (here referred as I-MC). Immune cells consist of effector T cells admixed with PDC and a, to our knowledge previously unreported, cell type resembling the so-called ''IFN-DC.'' Remarkably, immune cell infiltration was paralleled by increased immunogenicity of infected keratinocytes. To our knowledge, this finding establishes a unique model to underpin cellular mechanisms of the antiviral response to poxviruses.

Members of the Poxviridae family are particularly adept at escape from the host immune system by using different

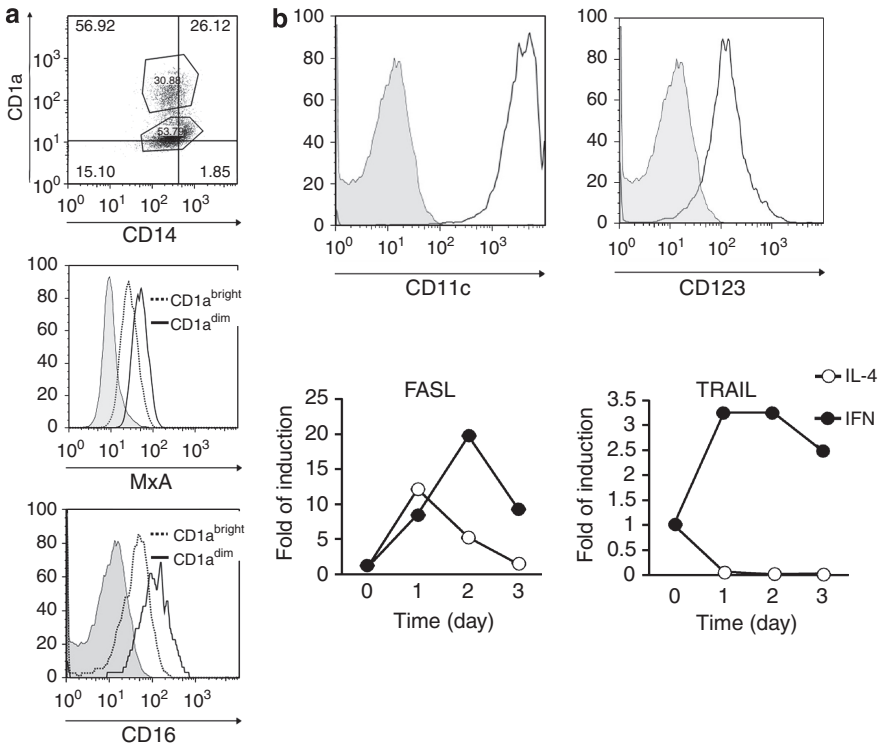

Figure 6. Characterization of monocyte-derived IFN-induced dendritic cells (IFN-DCs). IFN-DCs expressed variable levels of CD14 and CD1a. CD1a expression inversely correlated with the expression of MxA, CD16, and CD14 (**a**). IFN-DCs strongly express $CD123^+$ and $CD11c^+$ (**b**) and during their differentiation upregulate FasL and TRAIL that are at higher levels than those observed in classic monocyte-derived DCs obtained in the presence of GM-CSF and IL-4 (**b**).

strategies that sabotage components of the inflammatory response (reviewed in Moss *et al.*, 2000; Seet *et al.*, 2003). Genes encoded by poxviruses can target key host genes that ultimately reduce the immunogenicity of infected cells and hamper the recruitment of immune cells (Senkevich *et al.*, 1996; Krathwohl *et al.*, 1997; Damon *et al.*, 1998; Luttichau *et al.*, 2000). One of the remarkable findings of this study is represented by the detection of high numbers of PDCs in I-MC. Accumulation of PDCs in viral infections has been reported only in few conditions, represented by acute cutaneous varicella infection (Gerlini *et al.*, 2006) and hepatitis C virus positive hepatitis (Lau *et al.*, 2008). PDCs are capable of producing large amounts of type I IFNs and possess features of DCs (Grouard *et al.*, 1997; Cella *et al.*, 1999, 2000). They circulate through the blood (O'Doherty *et al.*, 1994; Sorg *et al.*, 1999) and colonize lymphoid organs, but are very scant in peripheral tissues, where they accumulate only in pathological conditions such as cancer and autoimmune diseases (Facchetti *et al.*, 2003; Colonna *et al.*, 2004). Clues for a direct role of PDCs in the response against viruses come from clinical and laboratory findings. PDCs sense viral RNA and DNA, because of their repertoire of Toll-like receptors (Kadowaki *et al.*, 2001; Krug *et al.*, 2001, 2004a, b; Lund *et al.*, 2003), and secrete high amounts of type I IFN in response to viruses (Cella *et al.*, 1999; Barchet *et al.*, 2005). The number of peripheral blood PDCs of patients infected by HIV, hepatitis B virus, and hepatitis C virus is severely diminished (Donaghy *et al.*, 2001; Duan *et al.*, 2004; Kanto *et al.*, 2004). In HIV, their reduced frequency correlates with disease progression and occurrence of opportunistic infections or Kaposi's sarcoma (Soumelis *et al.*, 2001).

PDCs might directly participate in the killing of infected cells via different effector mechanisms (Vermi *et al.*, 2004; Santoro *et al.*, 2005; Chaperot *et al.*, 2006; Hardy *et al.*, 2007; Stary *et al.*, 2007), or represent the primary source of proinflammatory chemokines and type I IFN (Penna *et al.*, 2002a, b). In this study, we found that only 50% of $BDCA2^+$ cells in I-MC express GrB, a key effector of the granule pathway primarily involved in the clearance of pathogen-infected cells. The significance of GrB expression and release by PDCs is still a matter of debate. It is of note that PDCs found in Imiquimod-treated skin cancer lack GrB (Stary *et al.*, 2007 no. 5462). Using qPCR of I-MC versus NI-MC and using antibodies directed against MxA and STAT1pY701, we found that IFN-α is locally produced and paralleled by a type I IFN gene expression signature. Type I IFN-targeted cells were represented by keratinocytes and inflammatory cells. Notably, infected keratinocytes composing I-MC lesions showed strong induction of MHC-I, MHC-II, TAP1, and TAP2, thus suggesting a contribution of this cytokine in increasing the immunogenicity of infected cells. Modulation of these molecules is also largely dependent on IFN-γ. Primary source for this cytokine can be represented by T cells and natural

killer cells infiltrating I-MC. Type I IFNs can modulate a plethora of immune cells with innate and adaptative functions including T cells (Kadowaki *et al.*, 2000; Biron, 2001; Blanco *et al.*, 2001; Krug *et al.*, 2004a; Jego *et al.*, 2005). Accordingly, in I-MC, we found that T cells were biased toward a Th1-type polarization as documented by the dominant expression of the transcription factor T-bet and abundant production of IFN-γ by qPCR. In addition, a large proportion of T cells was represented by $CD8^+$ cytotoxic T cells expressing perforin and GrB; notably, a fraction of them also coexpressed the activation marker CD30.

As additional proof that type I IFN represents a key cytokine in I-MC, we documented the existence of a cell population phenotypically resembling the so-called ''IFN-DC,'' a recently identified DC population that can be generated *in vitro* by type I IFN conditioning of peripheral blood monocytes (Santini *et al.*, 2000, 2003, 2005, 2009; Montoya *et al.*, 2002; Ferrantini *et al.*, 2008). In this study, we showed the $CD123^+CD11c^+$ cells found in I-MC lack lymphoid- and natural killer lineage markers and were positive for HLA-DR, CD40, CD14, CD16, and CCR7. A fraction of them also expressed CD83 and CD208, suggesting ongoing activation. According to their type I IFN-biological dependence, they also strongly reacted to anti-MxA and anti-STAT1pY701. Finally, by qPCR of RNA obtained from I-MC, we found high expression of Axl and Lox1, the two recently identified IFN-DC-associated genes (Scutera *et al.*, 2009; Parlato *et al.*, 2010). To our knowledge, the occurrence of a similar population resembling IFN-DC has never been documented in human tissues. We have been struck by the very consistent finding that these cells were strategically located in close proximity to active $caspase\text{-}3^+$ apoptotic keratinocytes, suggesting their direct involvement in the rejection process via a caspase-dependent mechanism. IFN-DCs can contribute in at least three different ways to the local immune surveillance to viruses. IFN-DCs can cross-present viral antigen to CD8 T cells, increase the immunogenicity of infected cells by producing IFNs, or exert effector functions through GrB and TRAIL (Lapenta *et al.*, 2006; Santini *et al.*, 2009). We were able to document GrB and TRAIL expression by I-MC-associated $CD11c^+CD123^+$ cells, and *in vitro* generated IFN-DCs showed a strong induction of TRAIL and FasL. Our data of IFN-DC occurrence in I-MC suggests a potential contribution of IFN-DCs in the immune response to MCV.

The histopathology of I-MC partially mimics the inflammatory reaction observed in Imiquimod-treated skin cancers. Imiquimod is a Toll-like receptor 7/8 agonist currently used with efficacy in the topical treatment of various skin cancers. Similarly to MC, clinical response is preceded by signs of local inflammation, and Imiquimod-induced regression is accompanied by a dense immune cell infiltration with sizeable numbers of cytotoxic T cells, PDCs, and a consistent population of $CD11c^+$ myeloid DCs (Stary *et al.*, 2007). Similar to I-MC, PDCs produce high amounts of type I IFN (Urosevic *et al.*, 2005) and the $CD11c^+$ myeloid DCs show a cytotoxic profile (Stary *et al.*, 2007). MC has been treated with Imiquimod and complete clearance have been obtained in 33% of the cases (Theos *et al.*, 2004), suggesting that appropriate boosting of local PDC is central to achieve clinical benefit.

In summary, this study provides a detailed analysis of cellular events associated with spontaneous regression of MC and proposes a relevant role for the crosstalk among two different DC subsets, namely PDCs and IFN-DCs. We speculate that one of the driver events in this scenario is represented by the recruitment of PDCs at the site of infection. Proinflammatory chemokines (Penna *et al.*, 2002a) produced by PDCs might attract other innate and adaptative immune cells. The availability of high amounts of type I IFN can favor the switch of monocytes to IFN-DCs, the Th1 polarization of T cells, and the activation of natural killer cells and CD8 T cells. This cellular environment would provide sufficient amounts of type I and II IFNs to increase the immunogenicity of infected keratinocytes by re-inducing MHC molecules that would present viral antigens and become more susceptible to recognition and elimination by CD8 T cells. Finally, although not mechanistically proved in this study, we favor the idea of an additional co-contribution of PDCs and IFN-DCs as innate effectors (Stary *et al.*, 2007). In this view, such a better knowledge of the cellular events leading to a productive immune surveillance against a virus-induced ''tumor-like'' condition might suggest strategies to properly boost the immune response against cancer.

MATERIALS AND METHODS

Patients and tissue samples

A total of 36 cases of MCV-induced skin lesions obtained from the Departments of Pathology or the Dermatopathology laboratories of three institutions (University of Brescia, Italy; University of California at San Francisco School of Medicine, San Francisco, CA, USA; and Friedrichshafen, Germany) were enrolled in the study. The diagnosis was based on classical histological MC features (Cribier *et al.*, 2001) and molecular analysis (Supplementary Material online and Supplementary Figure S1 online). Normal skin obtained from plastic surgery was used as controls. All tissue samples were fixed in 10% buffered formalin and embedded in paraffin. This study has been conducted in adherence to the Declaration of Helsinki Principles and use of the tissue material is regulated by the institutional board (Spedali Civili di Brescia). Written, informed patient consent was obtained.

Histochemistry and immunohistochemistry

Details are reported in Supplementary Material online. Briefly, 4 μm sections from formalin-fixed and paraffin-embedded tissues were used for immunohistochemical staining, using a set of antibodies whose specificities and main reactivity are reported in Supplementary Table S1 online.

Cell separation and culture

Human DCs. Peripheral blood mononuclear cells were isolated from buffy coats of healthy blood donors (through the courtesy of the Centro Trasfusionale, Spedali Civili di Brescia, Italy) by Ficoll gradient (Amersham Biosciences, Uppsala, Sweden). IFN-DC was performed as described (1) with only minor modifications in cell density. Monocytes were isolated by immunomagnetic selection (MACS Cell Isolation Kits; Miltenyi Biotec, Auburn, CA). Positively

selected $CD14^+$ monocytes were plated at the concentration of 1×10^6 cells ml^{-1} for indicated times in RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (Lonza, Basel, Switzerland) supplemented with 50 ng ml^{-1} GM-CSF and 20 ng ml^{-1} IL-4 (both Peprotech, Rocky Hill, NJ) to generate IL-4/DC, or with 100 ng ml^{-1} GM-CSF and 1,000 IU ml^{-1} IFN-α (Roferon, Roche, Milan, Italy) to generate IFN/DC.

Flow cytometry

Cells were washed and resuspended in phosphate-buffered saline containing 1% human serum and incubated with anti-CD1a, CD14, CD16, CD123, CD11c (BD PharMingen, San Jose, CA), and MxA (see Supplementary Table S1 online). Cells were analyzed by flow cytometry using a Partec flow cytometer (Munster, Germany).

Real-time PCR

For qPCR analysis, RNA was obtained from *in vitro* generated IFN-DC and fixed tissue from NI-MC and I-MC cases. Details are reported in Supplementary Material online.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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

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