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Original Article

Human cytomegalovirus impairs megakaryopoiesis by reducing c-Mpl expression and inducing apoptosis via the intrinsic pathway

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Abstract: Background: Human cytomegalovirus (HCMV) is a frequent life-threatening infection of immunocompromised individuals. A commonly observed clinical manifestation of active HCMV infection in these patients is thrombocytopenia. Clinical and laboratory evidence suggest that megakaryocytes are a target of HCMV infection, and that HCMV may actively suppress megakaryopoiesis contributing to loss of platelets. A mechanism for how HCMV impacts megakaryopoiesis has yet to be uncovered. Results: We found that HCMV infection impaired differentiation and proliferation of a cell line that is similar to megakaryocyte progenitor cells. Importantly, in this infection system, we observed a decrease in c-Mpl expression and induction of apoptosis by the mitochondrial-mediated intrinsic pathway. Furthermore, thrombopoietin treatment prevented HCV-induced apoptosis. Conclusions: Here, we have shown that HCMV inhibited megakaryocyte differentiation and proliferation with reduction in the c-Mpl positive cell population. HCMV also induced megakaryocyte apoptosis through the mitochondria-mediated intrinsic pathway. Therefore, HCMV induced thrombocytopenia is the consequence of interference with multiple processes during megakaryopoiesis.

Keywords: Cytomegalovirus, megakaryopoiesis, hematopoiesis, differentiation, proliferation, apoptosis, endomitosis

Introduction

Human cytomegalovirus (HCMV), a member of the beta-herpesvirus family, is one of the most common pathogens worldwide. Approximately 50 to 100% of adults are seropositive, which indicates exposure to HCMV infection at an early age [1]. The virus maintains a life-long presence due to its ability to establish a latent infection with the potential to reactivate. Although HCMV primary or reactivated infection is mostly asymptomatic or mild in immunocompetent individuals, it may lead to fatal complications in immunocompromised persons, such as allogeneic hematopoietic stem cell transplant (HSCT) or solid-organ transplant recipients and AIDS patients. It is also a major cause of congenital infection in newborns. Systemic HCMV infection in immunocompetent individuals have also been reported recently [2].

Thrombocytopenia is a regular hematologic presentation of active HCMV infection, especially in allogeneic HSCT recipients and newborns with congenital HCMV infection [3-6]. Large-scale clinical observation of autologous stem cell transplantation demonstrated that HCMV infection was one of two significant risk factors associated with the secondary failure of platelet engraftment [7] and persistent thrombocytopenia [8, 9]. Antiviral treatments, such as ganciclovir, have been shown to improve recovery of peripheral platelet count in these patients, highlighting the relationship between thrombocytopenia and HCMV infection [6, 8].

Circulating platelets come from mature megakaryocytes, which are differentiated cells of myeloid lineage found primarily in the bone marrow. HCMV establishes latent infection in myeloid progenitors, and the viral genome is maintained through differentiation. These cells serve as the reservoir for viral latency and as vehicles for viral dissemination [10-12]. Several observations suggest that inhibition of megakaryopoiesis accounts for HCMV-induced thrombocytopenia. In patients with congenital HCMV infection, examinations of bone marrow revealed a decreased number of megakaryocytes [5]. Hematopoietic proliferation was inhibited by HCMV infection in some studies, reducing the number of mature megakaryocytes and, therefore, platelets [13-16]. Furthermore, increasing clinical and laboratory evidence suggests that mature megakaryocytes are also susceptible to HCMV infection. Typical cytopathy of HCMV infection, such as intra-nuclear inclusion bodies, was found in megakaryocytes from newborns with congenital HCMV infection [17]. Viral DNA and gene expression were detected in megakaryocytes after challenge with HCMV *in vitro* [18, 19].

The underlying mechanistic events of HCMV-induced inhibition of megakaryopoiesis remain uncertain. Multiple steps are involved in megakaryocytic development and platelet production and each step could potentially be affected by HCMV. Most studies examining the effect of HCMV on megakaryocytes look at inhibition of proliferation, and do not focus on effects on differentiation or maturation processes. Precedent for HCMV interference with differentiation has been set in neural models of HCMV infection where the virus inhibits proliferation, differentiation, and maturation of neural stem and progenitor cells. In the present study, we explore the impact of HCMV infection on differentiation, proliferation, and survival of two megakaryocytic cell lines.

Materials and methods

Cells and culture conditions

Human megakaryoblastic cell line CHRF-288-11 was derived from a metastatic acute megakaryoblastic leukemia. M-07e is a human megakaryoblastic cell line [20], also from a leukemia, that is dependent on interleukin-3 or granulocyte macrophage colony-stimulating factor for growth [21]. Both cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA).

CHRF-288-11 cells were cultured as suspension in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in a humidified incubator with 5% carbon dioxide. Passage was done with fresh medium every 3 to 5 days. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF, 10 ng/ml; Peprotech, NJ, USA) was added to the culture medium for M-07e, while other conditions were similar to those for CHRF-288-11 [22].

Virus and infection

Human cytomegalovirus strain AD169 (4×10^4 $TCID_{50}$/ml) was used for all infections, and it was obtained from the Virology Laboratory at the Department of Microbiology, Xiang-Ya University, Hunan province, P.R. China. Inactivated HCMV was prepared by exposure of virus stock to ultraviolet (UV) light (Sylvania, G30W, Japan) at a distance of 15-20 cm for 30 minutes [23].

Differentiation of megakaryocytes and the endomitosis assay

Megakaryocyte cultures were seeded at 4×10^3 cells/mL in a 10-ml culture of growth medium supplemented with PMA (10 ng/ml dissolved in DMSO; Sigma-Aldrich, Saint Louis, MO, USA) and the indicated MOI of HCMV. Culture medium with PMA was refreshed every three days. At the indicated time points, cell morphology was determined by *in situ* Leishman's staining or cells were collected by treatment with 0.25% trypsin for the endomitosis assay. A parallel culture supplemented with dimethylsulfoxide (DMSO, 0.02% v/v; Sigma-Aldrich, Saint Louis, MO, USA) was used as vehicle control.

To visualize endomitosis, DNA content of individual cells was determined. Cells were fixed and permeabilized in 70% ethanol in phosphate buffered saline (PBS) at -20°C overnight and stored at -20°C for up to one week [24]. After washing with PBS, cells were stained with propidium iodide (PI, 0.04 mg/ml; Sigma-Aldrich, Saint Louis, MO, USA) in PBS for 30 minutes at 37°C. DNA content was measured by flow cytometry (EPICS Elite ESP, Beckman-Coulter,

Virgin Islands, USA) and analyzed with WinMDI software (version 2.9; Joseph Trotter). Histograms of DNA content show cells with various folds of ploidy (N, x-axis) relative to cell count (y-axis). The M1 region of the histogram defines the population with DNA content of 8-ploidy or above (≥8N) [25, 26], termed polyploid cells.

Proliferation assay

Megakaryocyte proliferation was determined by colony formation in a plasma clot system (1 ml): 4×10^2 megakaryocytes, IMDM 0.6 ml, 10% bovine serum albumin (GIBCO, Invitrogen, Grand Island, NY, USA) 0.1 ml, 10^{-3} M 2-mercaptoethanol (Sigma-Aldrich, Saint Louis, MO, USA) 0.1 ml, bovine plasma (GIBCO) 0.1 ml and 3.4 mg/ml calcium chloride (Sigma-Aldrich) 0.1 ml. Cells (4×10^3/ml into 10 ml-culture systems) [27] were challenged with HCMV at various MOI one hour before seeding into the semi-solid cultures. HCMV was added to the culture every day for 5 days. Infected cultures were incubated under standard conditions for 6 to 7 days, and then removed to score colony forming units with an inverted microscope. One CFU was defined as a cluster containing greater than 10 cells.

Detection of c-Mpl expression

An immunolabelling procedure for cell surface antigen was used for c-Mpl detection. Cells were infected every day with HCMV or UV-HCMV (MOI 10) or not infected for 5 days. 5×10^5 cells were collected and washed in cold PBS. Cells were incubated with APC-conjugated CD110 (c-Mpl) antibody (FAB1016A, R&D Systems, Minneapolis, MN, USA) at 4°C for 1 hour and examined by flow cytometry (FACSAria, BD Biosciences, San Jose, CA, USA). An APC-conjugated irrelevant IgG was used in parallel as an isotope control. Flow cytometric data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). Cells considered to be expressing c-Mpl are shown in region R1 of the histogram.

Apoptosis assays

Apoptosis of cells was tested with three different assays. Cell cultures (4×10^3 cells/mL in a 10-ml culture) were infected with the indicated MOI of HCMV or UV-HCMV every day or not infected for 5 days, and then harvested for apoptosis experiments. In order to determine the effect of thrombopoietin (TPO) on HCMV induced apoptosis of megakaryocytes, TPO (100 ng/ml; PeproTech, NJ, USA) was added to HCMV infected cultures (MOI 10) at day 5, collected the next day, and then examined for apoptosis with the Annexin V assay.

Annexiv V staining combined with PI staining was performed using the kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Apoptotic cells were detected by flow cytometry (EPICS Elite ESP, Beckman-Coulter, Virgin Islands, USA) within one hour of staining. Data were analyzed with WinMDI software (version 2.9; Joseph Trotter) and presented in density plots. Apoptotic cells are located in regions R2 and R3 on the histogram.

Caspase-3 is activated by both the intrinsic and extrinsic apoptosis pathways. Activated caspase-3 was detected in fixed megakaryocytes with a FITC -conjugated antibody provided by the kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Cells were examined by flow cytometry (EPICS Elite ESP, Beckman-Coulter, Virgin Islands, USA). An isotope control (irrelevant FITC-conjugated IgG) was run in parallel. Data was analyzed with WinMDI software (version 2.9; Joseph Trotter), and presented as histograms. Region M1 defines the area containing apoptotic cells with activated caspase-3.

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide) is a lipophilic fluorescent probe. Its fluorescence decreases when aggregates of JC-1 disassemble into monomers due to impairment of mitochondrial transmembrane potential, which is a sign of intrinsic apoptosis pathway activation. JC-1 staining was performed with the kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol, and examined by flow cytometry (EPICS Elite ESP, Beckman-Coulter, Virgin Islands, USA) within one hour. Data were analyzed with WinMDI software (version 2.9; Joseph Trotter) and presented as density plots shown. Apoptotic cells - indicated by loss of fluorescence - are shown in Region 2.

Statistical analysis

GraphPad Prism software (version 5.01, GraphPad software Inc.) was used for all statistical

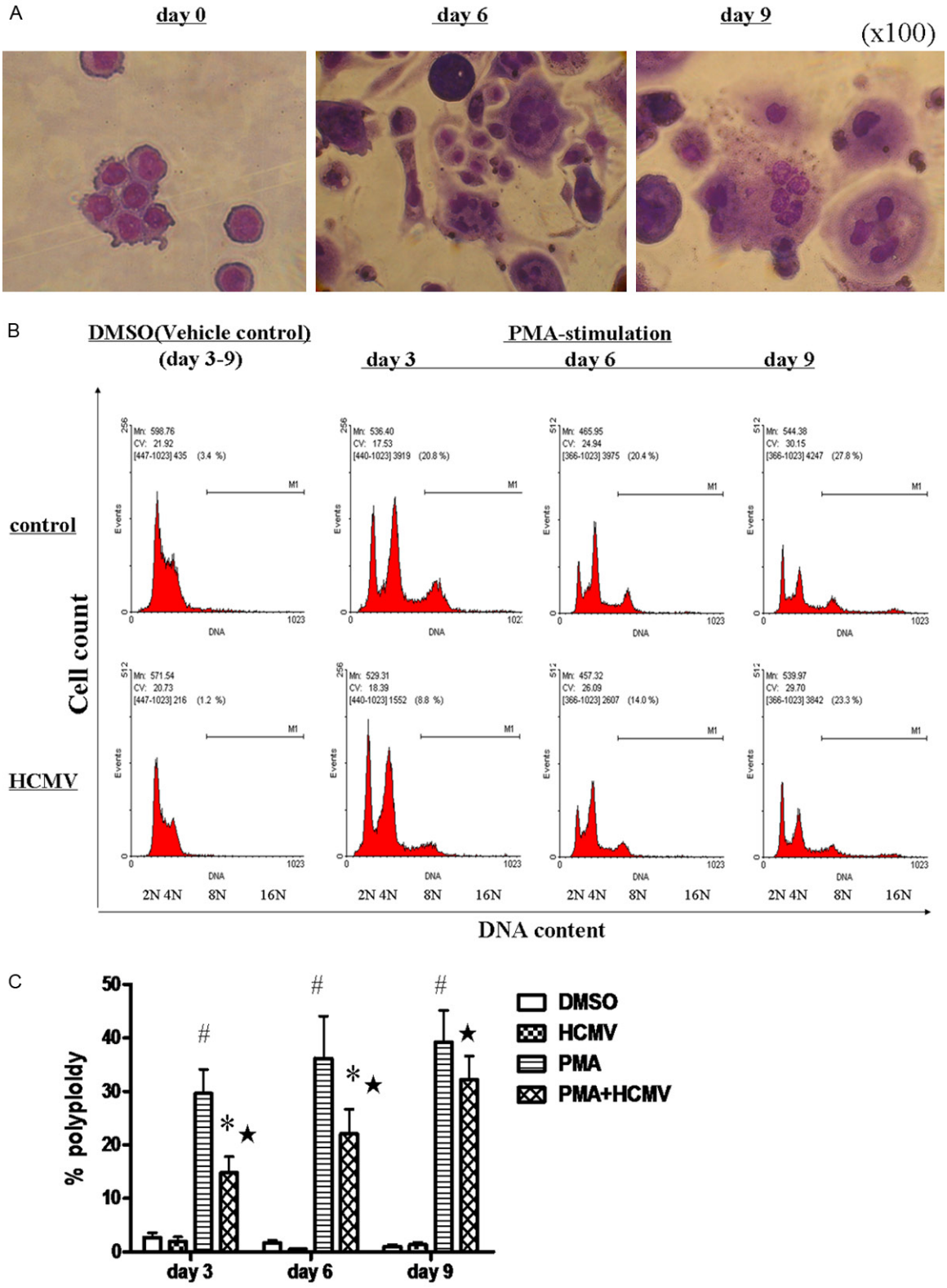

Figure 1. HCMV slows endomitosis of megakaryocytes. CHRF-288-11 megakaryocyte cells were exposed to HCMV (MOI 1) for 1 hr followed by PMA stimulation to induce differentiation. A. Morphology of uninfected PMA-stimulated cells from a representative experiment (Leishman's stain, 400×). Typical characteristics of megakaryocyte endomitosis were seen at day 6 and day 9. B and C. Polyploidization was determined by DNA content analysis using flow cytometry of propidium iodide stained DNA at the indicated time points. B. DNA content histograms for infected,

PMA-stimulated, and control populations from a representative experiment. Region M1 indicates polyploid cells (≥8N). PMA-stimulation led to polyploidy up to 16N in megakaryocytes (upper row), while HCMV reduced megakaryocyte polyploidization (lower row). C. Percent polyploid cells for each condition from 3 independent experiments were averaged and the data presented as mean percentage ± SEM. DMSO, DMSO treatment with no infection or PMA. HCMV, infected, but not treated with PMA. PMA, treated with PMA only. PMA+HCMV, infected and treated with PMA. Significance was calculated using two-way ANOVA followed by Bonferroni posttests. *P<0.05 versus PMA group, □P<0.05 versus HCMV group, and #P<0.05 versus DMSO group. HCMV, human cytomegalovirus; MOI, multiplicity of infection; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide.

analyses. The mean and standard error of the mean (SEM) were calculated for percent polyploidization, percent apoptotic cells, and counts of megakaryocyte colonies. Paired *t*-test was used for JC-1 assay data comparison, while two-way ANOVA and Bonferroni posttests were used for the rest of the data analysis. A difference was considered to be statistically significant when the *p*-value was less than 0.05.

Results

HCMV impairs PMA-induced megakaryocyte endomitosis

PMA, a phorbol diester, has been widely used in cell biology research to stimulate terminal differentiation of human hematopoietic progenitors *in vitro*. This model has been used for more than 20 years [28, 29], and it has been verified by multiple cell biological and morphological assays [30-33]. One hallmark of terminal differentiation is the phenomenon of endomitosis, where megakaryocytes become polyploid due to multiple duplications of the cellular genome. Endomitosis is easily assessed by staining of the DNA and determination of the total DNA content.

We induced differentiation with PMA of a megakaryocyte cell line (CHRF-288-11) that displays properties of the lineage-committed hematopoietic progenitors. Within one hour of exposure, the normally suspended cells were adherent and cell counts stabilized, indicating that they ceased proliferation. After nine days of PMA treatment, the cells increased in size (up to 5x larger than before PMA treatment), cytoplasmic volume, number of granules, and became polyploid (**Figure 1A**). Further analysis of DNA content showed the proportion of polyploidy cells (>8N) in PMA-treated cultures continuously increased over the course of the experiment (**Figure 1B**, top row). Ploidy of up to 64N was detected in some cells. DNA content in the DMSO control (**Figure 1B**, left column) or non-treated (data not shown) cells was mostly diploid (2N) with some tetraploidy (4N) observed. We note that shedding of platelets was rare. After nine days, cell senescence gradually increased.

To examine the effects of HCMV on differentiation of megakaryocytes, CHRF-288-11 cells were exposed to HCMV (MOI 1) one hour before treatment with PMA. The proportion of polyploid cells increased under these conditions; however, this occurred at a slower rate than in cells treated with PMA only (**Figure 1B**). The proportion of polyploid cells in HCMV-infected cultures compared to PMA-only cultures was reduced by 52%, 32% and 16% at day 3, 6, and 9 respectively (**Figure 1C**). The decrease in polyploid cells was specific to live virus while UV-inactivated HCMV did not affect the number of polyploid megakaryocytes (data not shown). Also, HCMV alone did not significantly affect the ploidy of cells without PMA stimulation (**Figure 1B**, bottom, left).

HCMV impairs proliferation of megakaryocytes

The colony-forming assay is a widely accepted method to observe proliferation of hematopoietic stem and progenitor cells. Megakaryocytes proliferated and began to form colonies after 2 days in suspension (data not shown). The count and average size of colonies increased up to day 6, after which it appeared that cells at the center of the colonies gradually began to die (data not shown). **Figure 2A** represents a typical view of colony morphology at day 7 under varying conditions.

Two megakaryocyte cell lines, CHRF-288-11 and M-07e, were tested to rule out cell line specific effects on proliferation. We note that colony counts of M-07e were slightly higher than that of CHRF-288-11 under all conditions (**Figure 2B**). Compared with uninfected controls, colony formation of HCMV infected cultures decreased by 26% in CHRF-288-11 cells and 23% in M-07e cells (**Figure 2B**), and the

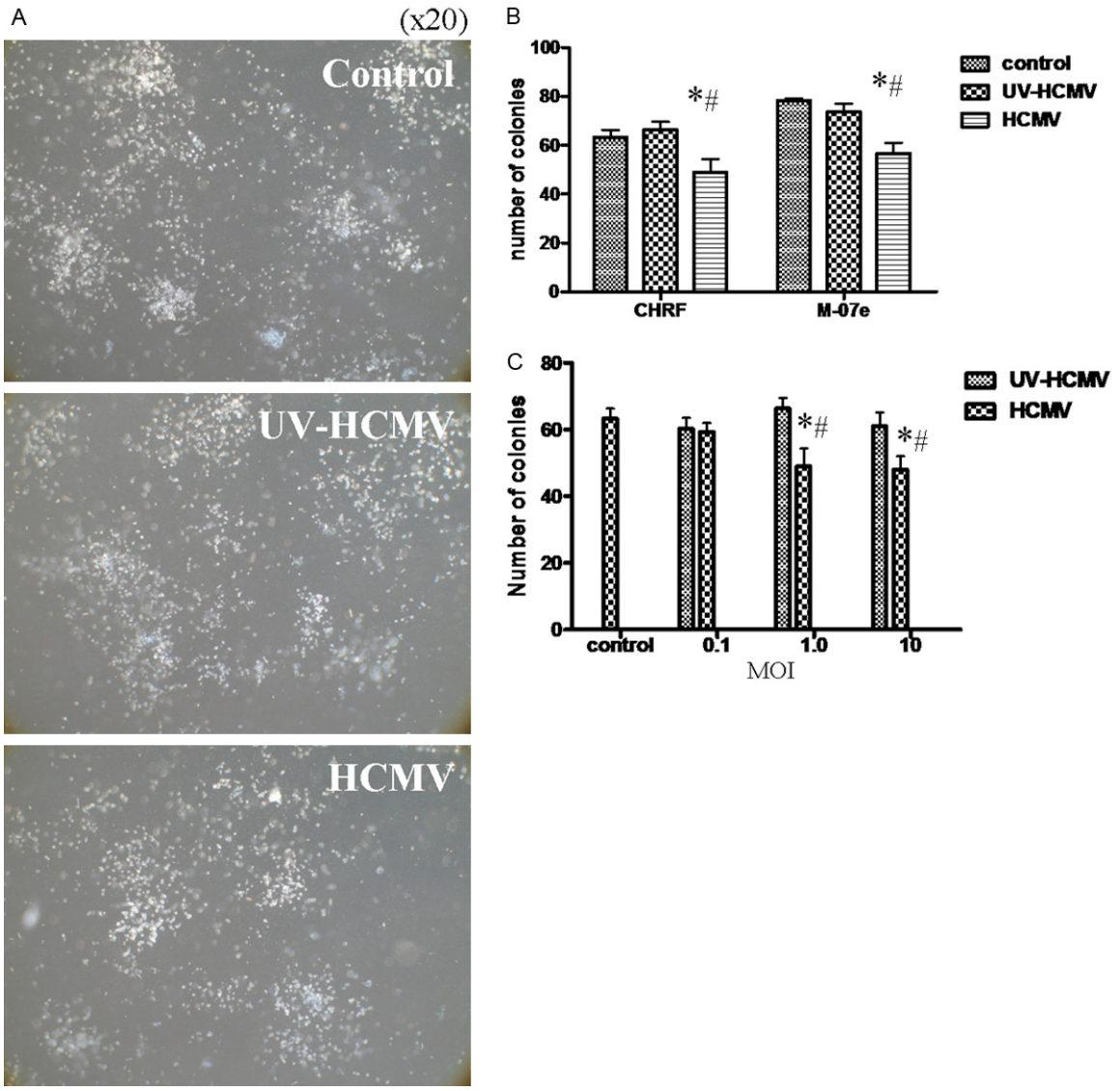

Figure 2. HCMV impairs proliferation of megakaryocytes. Two megakaryocyte cell lines, CHRF-288-11 and M-07e, were cultured in semi-solid medium to visualize proliferation by colony formation. Colonies, defined as clusters of ≥10 cells, were scored at day 7. A. Colonies of CHRF-288-11 cells that were uninfected or infected with UV-HCMV or HCMV (MOI 1) from a representative experiment are shown. The number and size of colonies decreased in HCMV infected culture. B. The number of colonies formed by CHRF-288-11 and M-07e under infected (UV-HCMV or HCMV (MOI 1)) or uninfected (control) conditions from at least 3 independent experiments was averaged. The data is presented as mean ± SEM. C. Colony formation of CHRF-288-11 cells infected with HCMV at various MOI. Results from 3 independent experiments are shown as mean ± SEM. Colony formation decreased in HCMV infected cultures with high viral load (MOI 1, 10), while there was no significant change in cultures with low viral load (MOI 0.1). Significance was calculated as described in **Figure 1**. *P<0.05 versus UV-HCMV group and #P<0.05 versus (uninfected) control group. UV-HCMV, ultraviolet-inactivated human cytomegalovirus.

average size of colonies (number of cells) decreased by roughly 30% in both cell lines (**Figure 2A**, bottom image and data not shown). The morphology of infected cells did not show any typical pathology of HCMV infection (inclusion bodies, enlarged nuclei or cell size) in either live culture or H&E staining (data not shown). Lower viral load (MOI 0.1) abolished the effect on proliferation, and a higher viral load (MOI 10) failed to enhance the effect (at least after 5 days) (**Figure 2C**). The decrease in colony number was not observed when cells were treated with UV-inactivated HCMV (**Figure 2A-C**).

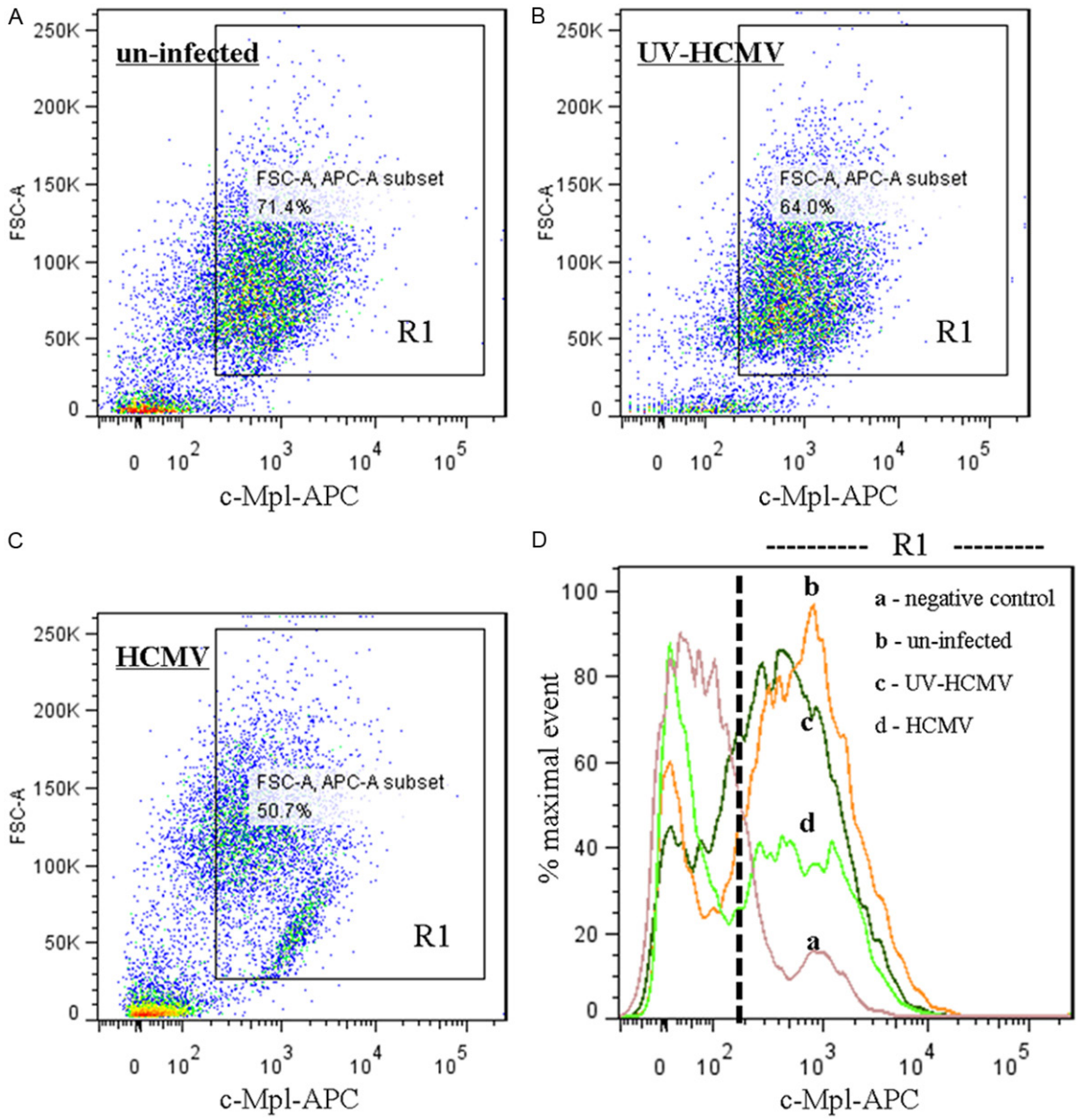

Figure 3. HCMV reduces the c-Mpl positive cell population. c-Mpl expression in CHRF-288-11 cultures was determined 5 days post infection (MOI 10) by staining cells with APC-conjugated c-Mpl antibody and detection by flow cytometry. Density plots of c-Mpl expression (c-Mpl-APC) in uninfected (A), HCMV infected (B), and UV-HCMV infected (C) megakaryocytes from a representative experiment are shown. Populations of c-Mpl positive cells are located in region R1. (D) The proportion of c-Mpl positive cells is reduced in HCMV-infected cultures. Results from two independent experiments were combined and are presented in a histogram of APC fluorescence. CHRF-288-11 cells were also stained with an APC-conjugated irrelevant IgG as an isotope control (negative control). FSC-A, area of forward scatter signals.

HCMV reduces the c-Mpl positive population of megakaryocytes

Expression of the hematopoietic growth factor receptor, c-Mpl, is essential at all stages of megakaryopoiesis. Congenital absence or functional impairment of the c-MPL gene results in severe amegakaryocytic thrombocytopenia or lethal pancytopenia due to an overwhelming reduction of megakaryocytes and multi-lineage hematopoietic stem and progenitor cells [34-36]. HCMV is known to down-regulate cellular surface receptors in other models of infection; thus, we tested the virus's ability to affect c-Mpl expression at the surface of megakaryocytes.

c-Mpl protein expression in UV-HCMV or HCMV infected megakaryocytes was determined 5

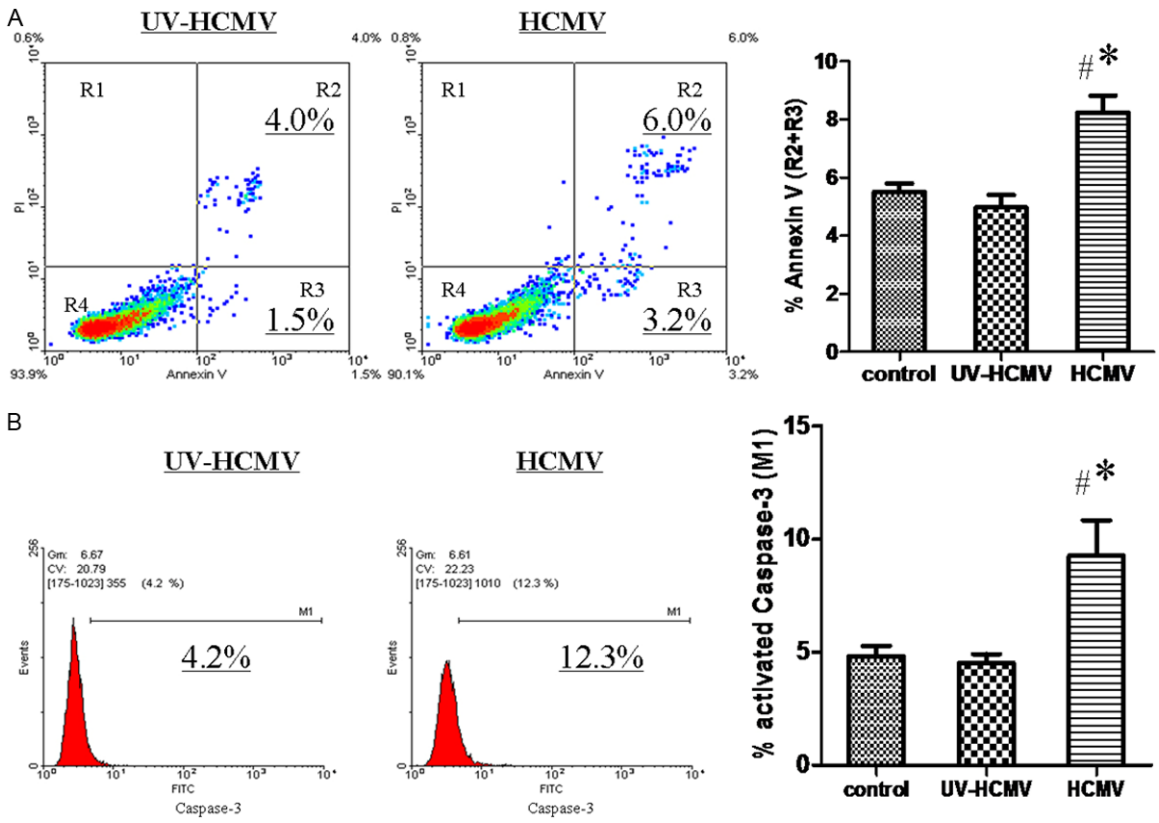

Figure 4. HCMV induces apoptosis in megakaryocytes. Five days post infection (MOI 10) CHRF-288-11 cells were harvested for analysis in apoptosis assays. A. Annexin V assay. Density plots from one representative experiment (left and middle) are shown. Apoptotic cells, defined as the total Annexin V positive population, are found in regions R2 and R3. Results from 3 independent experiments were averaged (right) and show a 49% increase of apoptotic cells in HCMV infected cultures compared to uninfected (control) cultures. UV-HCMV did not affect apoptosis. B. Activated caspase-3 detection. Representative histograms of activated caspase-3 from one experiment are shown (left and middle). Apoptotic cells are located in region M1. Activated caspase-3 showed an average increase of 94% among HCMV infected cells compared to uninfected cells (right, average of 3 independent experiments). Significance was calculated as described in **Figure 1**. *P<0.05 versus UV-HCMV group and #P<0.05 versus uninfected control group.

days after infection by flow cytometry. In routine cultures, 70% of uninfected CHRF-288-11 were c-Mpl positive (**Figure 3A**). HCMV infected cells exhibited a 29% decrease in the c-Mpl positive population, while UV-HCMV did not have a significant impact on c-Mpl expression (**Figure 3B**, **3C**). Also of note, the fluorescence intensity of c-Mpl staining of the positive population did not significantly change between uninfected and HMCV infected cells (compare R1 from **Figure 3A** and **3C**).

HCMV induces apoptosis in megakaryocytes via the mitochondria-mediated intrinsic pathway

Apoptosis of targeted cells is considered a major pathological event in active HCMV infection. Clinical and *in vitro* studies suggest that HCMV directly induces apoptosis in a spectrum of cells, including hematopoietic stem and progenitor cells [37-41] and megakaryocytes [40, 42, 43]. Using an Annexin V assay, apoptosis was detected in 5.5% of uninfected and UV-HCMV infected CHRF-288-11 cells (**Figure 4A**, left and right). In contrast, 9.2% of HCMV infected cells were apoptotic, representing a 49% increase over controls (**Figure 4A**, middle and right). Apoptosis as determined by activation of caspase-3 occurred in 4.2% of UV-HCMV and 12.3% of HCMV infected cells (**Figure 4B**, left and middle). That is a 94% increase in caspase-3 activated cells in the HCMV infected condition compared to controls (**Figure 4B**, right).

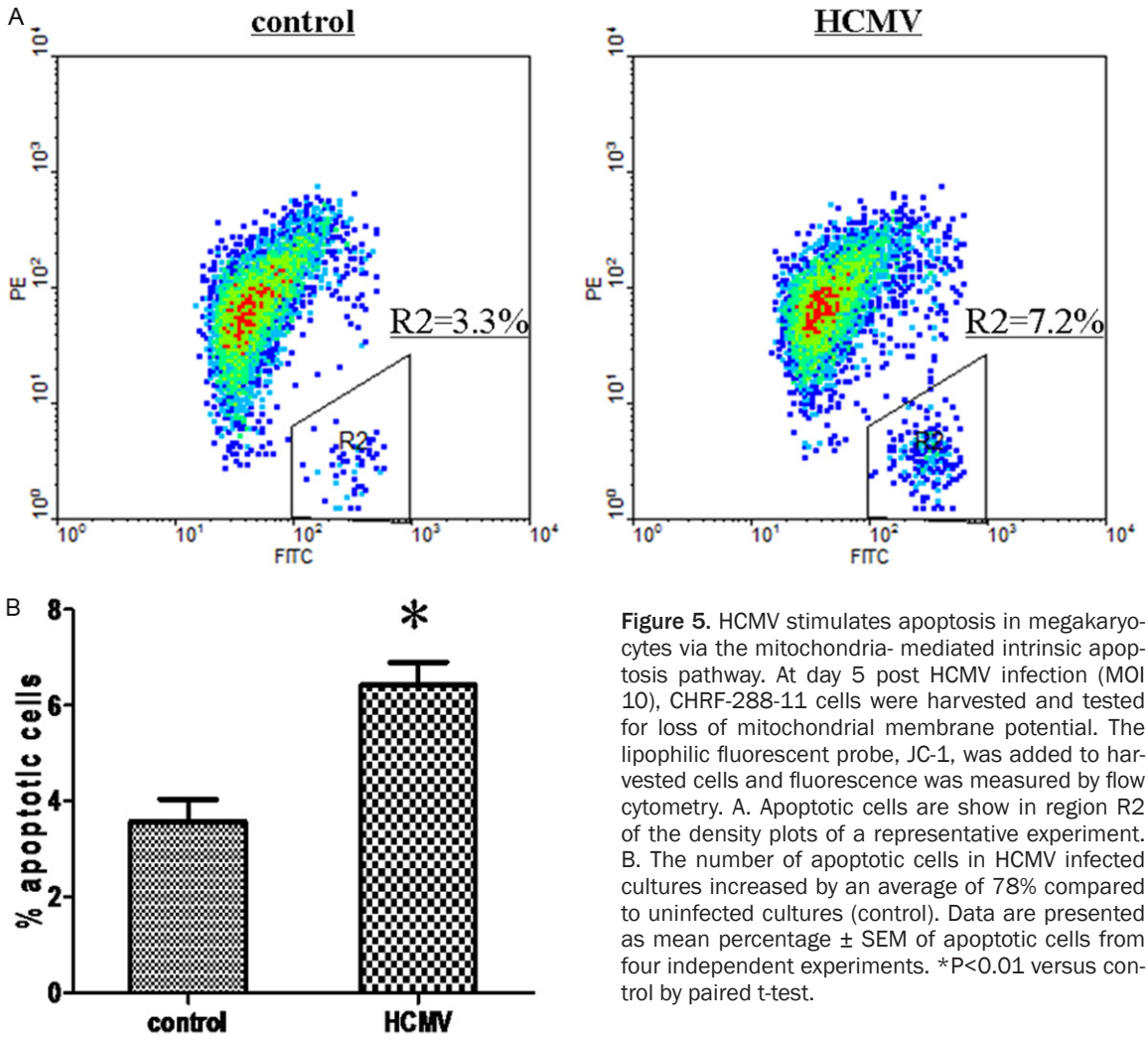

Figure 5. HCMV stimulates apoptosis in megakaryocytes via the mitochondria- mediated intrinsic apoptosis pathway. At day 5 post HCMV infection (MOI 10), CHRF-288-11 cells were harvested and tested for loss of mitochondrial membrane potential. The lipophilic fluorescent probe, JC-1, was added to harvested cells and fluorescence was measured by flow cytometry. A. Apoptotic cells are show in region R2 of the density plots of a representative experiment. B. The number of apoptotic cells in HCMV infected cultures increased by an average of 78% compared to uninfected cultures (control). Data are presented as mean percentage ± SEM of apoptotic cells from four independent experiments. *P<0.01 versus control by paired t-test.

Mitochondrial permeabilization followed by cytochrome C release into the cytoplasm is a characteristic event in the intrinsic apoptotic pathway. Impaired mitochondrial transmembrane potential is a sign of permeabilization, and can be measured using the lipophilic fluorescent probe, JC-1. JC-1 fluorescence decreases as the membrane potential is lost. In the uninfected control, approximately 3.6% of cells had impaired mitochondrial membrane potential (**Figure 5A**), while HCMV infected cultures showed an average of 6.3% (a 78% increase) (**Figure 5B**).

Thrombopoietin treatment prevents HCMV induced apoptosis of megakaryocytes

Thrombopoietin (TPO) is a key hematopoietic/megakaryopoietic regulating growth factor, and it is the ligand of the c-Mpl receptor. In addition to its role in regulating megakaryopoiesis, it provides protection against apoptosis in neural, cardiac, hematopoietic stem and progenitor cells, and megakaryocytes *in vitro* [44-51]. After short-term TPO treatment, apoptosis of HCMV infected CHRF-288-11 cells was determined using the Annexin V assay. Among non-TPO treated controls, only HCMV infected cultures displayed increased apoptosis as seen previously (**Figure 6A**, upper row). TPO treatment inhibited the apoptotic effect of HCMV infection, bringing apoptotic levels down to that of the uninfected or UV-HCMV infected cultures (**Figure 6B**).

Discussion

Under normal circumstances thrombocytopenia should produce signals to stimulate megakaryocyte terminal differentiation (and endomi-

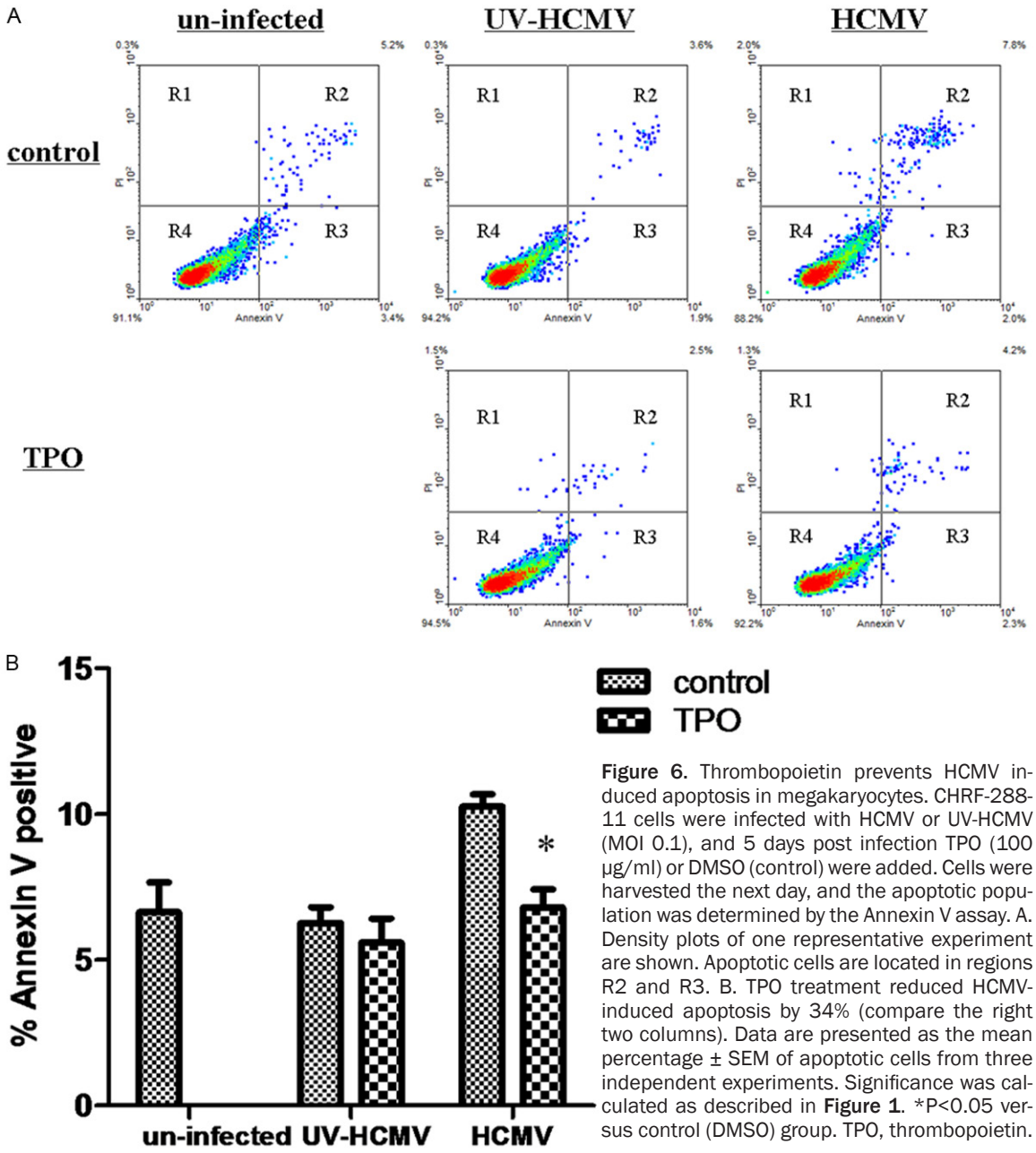

Figure 6. Thrombopoietin prevents HCMV induced apoptosis in megakaryocytes. CHRF-288-11 cells were infected with HCMV or UV-HCMV (MOI 0.1), and 5 days post infection TPO (100 μg/ml) or DMSO (control) were added. Cells were harvested the next day, and the apoptotic population was determined by the Annexin V assay. A. Density plots of one representative experiment are shown. Apoptotic cells are located in regions R2 and R3. B. TPO treatment reduced HCMV-induced apoptosis by 34% (compare the right two columns). Data are presented as the mean percentage ± SEM of apoptotic cells from three independent experiments. Significance was calculated as described in **Figure 1**. *P<0.05 versus control (DMSO) group. TPO, thrombopoietin.

tosis) driving platelet production. Yet, in an immunocompromised person active HCMV infection induces and allows sustained thrombocytopenia, which can lead to serious complications if left untreated. Although it is known that HCMV can infect megakaryocytes and their progenitors, the molecular mechanisms governing HCMV's ability to cause thrombocytopenia are still unidentified. We employed cell lines with properties similar to megakaryocyte progenitors that can be chemically induced to terminally differentiate into mature megakaryocytes. The effects of HCMV infection of these cells on various megakaryocyte phenotypes were evaluated.

When differentiation was stimulated with PMA normal megakaryocyte endomitosis was observed in uninfected cells, validating the use of this system. Our results suggest that HCMV infection impairs megakaryocyte differentiation induced by PMA, resulting in reduced endomitosis. Impairment of megakaryocytic differentiation has been seen with other viral

infections [34, 52]; however, this is the first demonstration of inhibition of late-stage differentiation of committed megakaryocytic progenitors by HCMV.

In agreement with previous reports of HCMV infection of undifferentiated bone marrow hematopoietic stem cells and cord blood megakaryocyte progenitors [15, 53]; proliferation was inhibited by HCMV infection in our cell culture system. This effect was not cell line specific as both CHRF-288-11 and M-07e cell proliferation were similarly reduced. We observed a plateau of inhibition at MOI 1. This effect is likely due to limited observation of the infected cultures. Unfortunately, the *in vitro* colony-formation assay is not suited for long term experiments since megakaryocytes begin to lose viability after 7 days.

Both of the phenotypes described above could contribute to HCMV induced thrombocytopenia. Several possibilities exist to explain how HCMV might affect differentiation and proliferation at the molecular level. Reduced proliferation and differentiation of megakaryocytes required the presence of live virus in this study, suggesting that viral gene expression or replication is essential. Some HCMV viral gene products have already been implicated in inhibition of proliferation. Studies from other groups showed that expression of HCMV matrix protein pp71 in hematopoietic cells resulted in inhibition of cellular proliferation [54]. Also, impairment of cellular functions and apoptosis induced by HCMV infection may involve activation of several downstream molecular events [55, 56]. Viral and molecular targets contributing to the effect on differentiation seen here are more obscure. This is, in part, due to the fact that the events of terminal differentiation and endomitosis are not well characterized at the molecular level. Though, the possibility remains that certain HCMV gene products could directly affect cellular gene expression associated with megakaryocyte differentiation, or they could directly block cellular signal transduction induced by growth factors or cytokines to prevent differentiation.

HCMV has been reported to cause the down-regulation of important cellular receptor proteins in other systems, for example HLA class II antigen in endothelial cells and EGFR in fibroblasts [57-64]. Our data demonstrate that protein expression of a key receptor for progression of megakaryopoiesis, c-Mpl, was significantly reduced in the total megakaryocyte population after HCMV challenge. This is a critical finding to help explain the inhibitory effects seen on differentiation and proliferation. In a clinical setting, reduction of c-Mpl would lead to a poor response to TPO, the major ligand responsible for inducing platelet production. The underlying molecular mechanism of this inhibitory effect on c-Mpl expression is still uncertain. Evidence from other studies suggest that viral transcription or expression, such as US3, UL36, UL37, UL38, IE and E proteins, may be involved in inhibition of expression of various regulatory genes [63, 65, 66].

Another obvious potential cause for both reduced differentiation and proliferation is induction of cell death by HCMV infection. This may include autophagy, necrosis, or apoptosis. Apoptosis was increased in our HCMV infected megakaryocytes, interestingly, by a mitochondrial-mediated intrinsic pathway. While mitochondria- mediated apoptosis has been found to be a major pathological event in other herpesvirus infections [67-70], HCMV-induced intrinsic apoptosis has seldom been reported. In fact, the extrinsic apoptotic pathway has been shown to be activated in HCMV-infected hematopoietic progenitor cells, which is mainly triggered by the immune response and has been considered a mechanism to prevent spread of HCMV in the body [55, 71, 72]. The activation of the intrinsic apoptosis pathway by HCMV in megakaryocytes is a novel finding of this study and will be pursued further.

Finally, we demonstrate that treatment with TPO eases HCMV induced apoptosis of megakaryocytes. This is not completely without precedent as previous studies have also reported TPO's protective effect against apoptosis and other harmful conditions in other systems [46, 47, 50]. Our findings may serve as scientific support for the potential clinical application of TPO in HCMV-induced thrombocytopenia [73].

Conclusions

In summary, we found that HCMV inhibited megakaryocyte differentiation and proliferation with reduction in the c-Mpl positive cell population. HCMV also induced megakaryocyte apoptosis through the mitochondria-mediated intrin-

sic pathway. Therefore, HCMV induced thrombocytopenia is the consequence of interference with multiple processes during megakaryopoiesis. Prophylactic or pre-emptive therapies for HCMV reactivation and direct treatment against viral replication are essential for clinical management. Growth factors such as TPO could significantly reduce HCMV induced apoptosis on megakaryocytes, and it has potential for use in conjunction with anti-viral therapy to reverse HCMV induced thrombocytopenia.

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Disclosure of conflict of interest

None.

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