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Expression of Werner and Bloom syndrome genes is differentially regulated by *in vitro* HIV-1 infection of peripheral blood mononuclear cells

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SUMMARY

In HIV infection, continuous immune activation leads to accelerated ageing of the adaptive immune system, similar to that observed in elderly people. We investigated the expression of WRN and BLM (genes involved in disorders characterized by premature ageing, genomic instability and cancer predisposition) in peripheral blood mononuclear cells (PBMC) activated *in vitro* with phytohaemagglutinin (PHA) and infected with different HIV-1 strains. The steady state levels of mRNA were analysed by reverse transcription-polymerase chain reaction (RT-PCR), and protein expression was assayed using immunocytochemistry and Western blot techniques. In uninfected PBMC, PHA stimulation induced an increase in BLM mRNA and protein expression, while WRN expression remained virtually unchanged. When PBMC were infected *in vitro* with a lymphotropic HIV-1 strain, the level of BLM mRNA showed a peak at 24 h of infection, followed by a decline to uninfected culture levels. A similar result failed to be seen using an R5-tropic HIV-1 strain. In accordance with mRNA expression, in HIV-infected cultures PBMC were stained more frequently and more intensely by a BLM-specific antibody as compared to uninfected cultures, staining peaking at 24. Conversely, WRN expression was not modulated by HIV-1. The proportion of cells showing BLM up-regulation, established by immunocytochemical staining, was much greater than the proportion of productively infected PBMC, as established by proviral DNA measurement. This result indicates that BLM up-regulation is probably a result of an indirect bystander cell effect. Activation of the BLM gene in infected PBMC suggests that premature ageing could be a further immunopathogenetic mechanism involved in HIV-induced immunodeficiency, and points to a possible new candidate target for innovative therapeutic intervention.

Keywords ageing BLM HIV-1 infection mRNA expression T lymphocytes

INTRODUCTION

During antigen-induced T lymphocyte activation dramatic changes in the pattern of gene expression occur. These changes take place predominantly within 8 h of activation, but gene expression returns to levels similar to those of resting cells within 48 h of exposure to antigens, as shown by microarray analysis [1]. Not surprisingly, genes associated with cell division (cyclin, cyclin-dependent kinase and DNA polymerase) in activated T cells are expressed at higher levels than in resting T cells. On the contrary, resting cells express higher levels of genes thought to inhibit cell division and of genes coding for a number of cytokine receptors [2]. A class of genes known to be differentially regulated during cell cycle is the family of RecQ helicases that includes *Escherichia coli* RecQ, *Saccharomyces cerevisae* Sgs1, *Schizosaccaromyces pombe* Rqh1 and five human RecQ helicases, namely WRN, BLM, Q1/RecQL (RecQL1), Rothmund–Thomson's syndrome gene product (RecQL4) and RecQL5 [3–11]. In human B cells transformed by Epstein–Barr virus (EBV) or stimulated with phorbol myristic acetate (PMA), human fibroblasts and umbilical vein endothelial cells transformed by simian virus 40 (SV40), the expression of WRN and BLM is up-regulated [12]. Moreover, immunocytochemical staining of proliferating fibroblasts and B-lymphoblastoid transformed cells show higher levels of WRN helicase than normal cells [13]. WRN and BLM genes are involved in genetic disorders, characterized by premature ageing, genomic instability and cancer predisposition [14,15]. The aberrant cellular and clinical phenotypes (Werner and Bloom syndromes) arise from defects in important DNA metabolic pathways, such as those used for replication, recombination or repair [16–18].

Experimental evidence suggests some analogies of T cell dynamics between advanced ageing and HIV infection. The progressive loss of naive T cells within the $CD8^+$ subset [19–23], the

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expansion of $CD8^+CD28^-$ T cells [24], as well as the restriction of the $CD8^+$ T cell repertoire [25,26] suggest a typical perturbation of the T cell subpopulations that is seen in both HIV disease and advanced ageing. Moreover, a decreasing number of recent thymic emigrants, identified as T cell receptor excision circles (TRECs) [27–29], have been observed with ageing and in HIV-infected individuals, while highly active antiretroviral therapy (HAART) treatment leads to an increased thymic output [29,30]. Therefore, there is a developing hypothesis that HIV-1 infection leads to an acceleration of the adaptive immune system ageing process, resulting in a premature exhaustion of immune resources and leading eventually to the onset of immunodeficiency [31]. Again, postmortem studies in clinically unaffected joints from AIDS patients provided histological evidence of premature ageing [32].

On the basis of these data, we decided to investigate the expression of WRN and BLM genes in healthy donor peripheral blood mononuclear cells (PBMC) after *in vitro* infection with HIV-1. To this end, we first analysed PBMC stimulated or not with phytohaemagglutinin (PHA). The same analysis was performed on PHA-stimulated PBMC after infection with HIV-1, using two viral strains with different tropism. The steady state levels of mRNA for WRN and BLM, as well as protein expression, were evaluated by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR), immunocytochemical staining and Western blot, respectively.

MATERIALS AND METHODS

PBMC separation and stimulation

PBMC from healthy donors were obtained by Ficoll/Hypaque (Pharmacia, Sweden) density centrifugation, washed three times with phosphate-buffered saline (PBS) and stimulated using 1 μg/ml PHA (GIBCO, Carlsbad, CA, USA) in RPMI-1640 medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml.

HIV-1 infection

Two different strains of HIV-1 were used for infection: a laboratory strain from a primary isolate, described previously as a lymphotropic X4 strain [33] and the macrophage-tropic HIV-1BaL R5 strain [34]. Viral stocks were obtained by infecting PHA blasts or acutely infected macrophage cultures, respectively.

PBMC were stimulated with PHA (1 μg/ml) for 2 days in RPMI-1640 medium containing 10% FCS, 2 mM l-glutammine, 100 U penicillin/ml and 100 μg streptomycin/ml (2×10^6 cells/ml) (GIBCO). Activated PBMC (2×10^6/ml) were then exposed to a fixed virus amount (2 ng/ml of p24) and incubated at 37°C with occasional mixing for 2 h. The cells were centrifuged, washed twice with PBS and reseeded at 2×10^6 cells/ml with interleukin (IL)-2 (50 IU/ml, Boehringer Mannheim, Ridgefield, CT, USA). At the indicated times, replicate aliquots of 500 000 cells from each experimental condition were spotted onto glass and fixed with 4% paraformaldehyde for immunocytochemical analysis. The remaining cells were pelleted and used for nucleic acid extraction.

Quantification of virus replication

Quantification of virion-associated p24 antigen released into culture supernatants was used to monitor virus replication. Samples of 200 μl were taken from each culture at the indicated times (0, 4 h, 24 h and 48 h) and stored at –70°C until completion of the experiment. Assays for p24 antigen were performed using the enzyme-linked immunosorbent assay (ELISA) method (Vironostika HIV-1 antigen; Sanofi-Biomérieux, Marcy l'Etoile, France). Supernatants from uninfected culture wells were included as negative controls. The concentration of p24 released in the culture supernatants was calculated according to the manufacturer's instructions, with the aid of values of the viral samples with a standard p24 antigen curve.

DNA extraction and determination of proviral HIV-1 copies

At the indicated times, total HIV-1 DNA was extracted from a known amount of PBMC (ranging from 4×10^6 to $4{\cdot}7 \times 10^6$ cell) using a QIAamp DNA kit (Qiagen Gmbh, Hilden, Germany), as specified by the manufacturer. Determination of proviral HIV-1 DNA copies was performed by real-time quantitative PCR targeting the *pol* region, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Primers and probe sequences were as follows: forward HIVDN Ap1–5′TGGCATGGGTACCAGCACA, reverse HIVDN Ap2–5′CTGGCTACTATTTCTTTTGCTA, probe (FAM) HIVDNA-TTTATCTACTTGTTCATTTCCTCCAAT TCCTT (TAMRA).

Amplification was performed in a 50 μl reaction mixture containing 10 μl of template, 25 μl of 2× *Taq*Man Universal PCR Master Mix (Applied Biosystems), 300 nM of each primer and 200 nM of probe. Following a uracil N-glycosylase (UNG) incubation for 2 min at 50°C and activation of the Ampli*Taq* Gold for 10 min at 95°C, 45 cycles of amplification (15 s at 95°C and 1 min at 65°C) were performed. The standard curve was prepared using serial dilutions of DNA from 8E5LAV cells, which contain 1 DNA proviral copy per cell. The standard curve was created automatically using ABI 7700 Sequence Detection System software by plotting the Ct values against each standard of known concentration. The analytical sensitivity of the system is estimated to be about 10 copies/reaction, with a linear range from 10 to 10^6 copies/reaction. The Ct values from unknown samples were plotted on the standard curve and the number of copies per reaction extrapolated. Samples were considered negative if the Ct was = 45. The reported proviral HIV-1 copy number relates to the amount seen in 1×10^6 PBMC.

RT-PCR

At the indicated times, 1×10^7 cells from each experimental condition were processed for total RNA extraction with triazol (GIBCO), as recommended by the manufacturer.

RNA RT was performed with M-MuLV reverse transcriptase according to standard procedures [35]. The primers and probes for BLM and WNR were chosen to represent the coding region of the genes. WRN forward primer: 5′-GGATCAGCACAGTCA GAAAATGTTCT-3′; reverse primer: 5′-GGATAGATTCAG TTTCCTAAGTTCACC-3′; probe: 5′-CCAATCGTTGCACT TACTGCTACTGC-3′. BLM forward primer: 5′-CTCCAGGCG AGAATGTGACACCATGGC-3′; reverse primer: 5′-CACAAG AAACATCTGGGTGTTTCTTAC-3′; probe: 5′-ATCTGTGGA GGGTTACTACCAAGAATCTGGC-3′. Amplification conditions: WRN, BLM: 30 cycles (94°C for 1·30 min, annealing at 60°C for 1 min, extension at 72°C for 1·30 min) followed by 5 min at 72°C. Semiquantitative PCR reaction was performed by the limiting dilution approach, using a 10-fold dilution of starting

cDNAs. As a measurement of internal control, β-actin gene expression was used [36].

Amplicons were separated by electrophoresis on a 1·2% agarose gel, transferred to nitrocellulose filter by Southern blot and assayed by hybridization with specific [^{32}P]γ-ATP radiolabelled probes. Quantitative data were obtained after transformation of the data from the gels by densitometric scanning of the autoradiographs. The intensities of the bands were calculated for each gene at the various dilutions, using the last dilution with a detectable hybridization signal of the relevant gene for each calculation. The results were expressed as a ratio to β-actin mRNA. The results are shown as mean ± s.d. of multiple experiments, performed on PBMC from at least five donors, as specified in the figure legends.

Immunocytochemical analysis and Western blot

Immunocytochemical staining of PBMC was performed using anti-BLM and anti-WRN polyclonal IgG raised in rabbit (Abcam, USA) as primary antibodies, diluted 1 : 50. A biotinylated goat antirabbit IgG was used as a secondary antibody, followed by the addition of preformed avidin–horseradish peroxidase complex (Biospa, Milano, Italy). The immunoreaction product was revealed using 3-3′ diaminobenzidine (DAB) as a chromogen substrate. Negative control staining was performed by omitting the primary antibody. Endogenous peroxidase activity was blocked by 3% H_2O_2. Cells were counterstained in Mayer's acid haemalum. Analysis was performed using a light microscope at 40× magnification, and quantification of stained cells (expressed as mean percentage ± s.d.) was performed by counting WRN and BLM positive cells over 500 total cells by two independent investigators in a blind fashion. Immunocytochemical analysis was carried out on PBMC obtained from five different donors.

Western blot was performed by standard procedures, by staining the blotted proteins with the same antibodies used for immunocytochemical analysis, diluted 1 : 500.

Statistical analysis

Mean values for mRNA and protein expression levels were compared using Student's *t* test. Differences with *P*-values < 0·05 were considered statistically significant.

RESULTS

To establish whether the expression of WRN and BLM genes is modulated in normal lymphomonocytes by mitogen stimulation, PBMC from healthy donors were cultivated in the presence or absence of 1 μg/ml PHA. After 24 and 48 h of treatment, RNA was extracted and RT-PCR was carried out by limiting dilution analysis, as described in Materials and methods.

In Fig. 1a, autoradiographs obtained from serial 10-fold dilutions of cDNAs amplified with primers specific for WRN and BLM are shown. As a control, β-actin cDNA was tested in parallel. A clear increase in BLM mRNA expression was observed after 24 h of stimulation that was even more evident after 48 h. Only slight differences were observed for WNR at both 24 and 48 h of exposure to PHA. The corresponding mRNA levels, expressed as a ratio to β-actin gene expression, are shown in Fig. 1b. Compared to untreated cultures, BLM mRNA steady state levels were significantly increased in PHA-stimulated cultures. The increase observed after 48 h of stimulation was higher (2·3-fold; $P = 0{\cdot}025$ *versus* unstimulated PBMC) than that observed at 24 h (1·8-fold; $P = 0{\cdot}034$ *versus* unstimulated PBMC) (Fig. 1b). With regard to WRN mRNA, increased levels of mRN were detected in PHA-stimulated compared to unstimulated PBMC, but the differences were not statistically significant (24 h: 1·4-fold; $P = 0{\cdot}101$; 48 h: 1·2-fold; $P = 0{\cdot}135$) (Fig. 1b).

In parallel, WRN and BLM protein expression was analysed by immunocytochemistry. Results obtained with this analysis matched the mRNA expression results. After 48 h of culture PBMC were positive for WRN protein staining, and only a slight increase of staining was observed in PHA-stimulated cells. On the contrary, weak staining for BLM protein was observed in untreated cells, while PHA-activated cells showed intense staining for BLM protein (Fig. 1c).

In order to analyse the effect of HIV infection on WRN and BLM gene expression, PBMC from healthy donors were infected with HIV-1 strains of different tropisms, namely a lymphotropic X4 strain (Is13) and a macrophage-tropic R5 strain (BaL).

PBMC were stimulated for 48 h with PHA infected in parallel with each of the two strains, and then analysed for the expression of WRN and BLM mRNA and protein levels. Viral replication was confirmed by measuring p24 release in supernatants (Fig. 2a,d). It should be noted that HIV-1 BaL shows a lower level of virus replication compared to HIV-1 Is13.

WNR and BLM mRNA levels in PBMC infected with HIV-1 Is13 are shown in Fig. 2b,c. The results indicated that WNR mRNA levels were not affected by the infection. In contrast, BLM mRNA levels were significantly increased at 24 h postinfection in PBMC infected with HIV-1 Is13 (twofold, $P = 0{\cdot}04$ *versus* uninfected cells), followed by a decline to levels similar to control uninfected cultures at 48 h (Fig. 2c).

Immunocytochemical analyses for WRN and BLM proteins were performed in the same samples and confirmed the effect of HIV-1 Is13 on WRN and BLM gene expression (Fig. 3). According to mRNA expression, WRN protein synthesis was virtually unchanged by HIV-1 infection at all time-points considered (Fig. 3a–c). However, HIV-1 Is13 induced an increased expression of BLM protein, detected as both higher intensity immunostaining (Fig. 3d–f) and increased proportion of positive cells (31 ± 4% *versus* 18 ± 2%, i.e. 1·7-fold increase, $P < 0{\cdot}05$) at 24 h postinfection. The increase was confirmed by Western blot analysis (data not shown). A lower increase of BLM immunostaining was observed at 48 h postinfection, albeit to a lesser extent (data not shown).

To establish whether the increase of BLM expression in PBMC cultures infected with the X4 HIV-1 strain was directly connected to HIV-1 infection, we determined the proportion of virus-infected cells in these cultures by real-time quantitative PCR for proviral HIV-1. According to data from the literature [37], we can assume that in our experimental conditions (i.e. at low multiplicity of infection) a single provirus was present in each infected cells at the considered time-points. The results indicated that after 24 h of infection approximately 6000 cells/1 × 10^6 PBMC resulted positive for proviral DNA, and a further increase was observed at 48 h postinfection (27 000 cells/1 × 10^6 PBMC). Therefore, the proportion of virus-infected cells was much lower than the proportion of BLM-expressing cells, suggesting that the increased BLM expression was an indirect, rather than a direct, effect of HIV-1 infection.

In contrast with the findings obtained with the X4 strain, PBMC infected with the R5 HIV-1 strain showed levels of

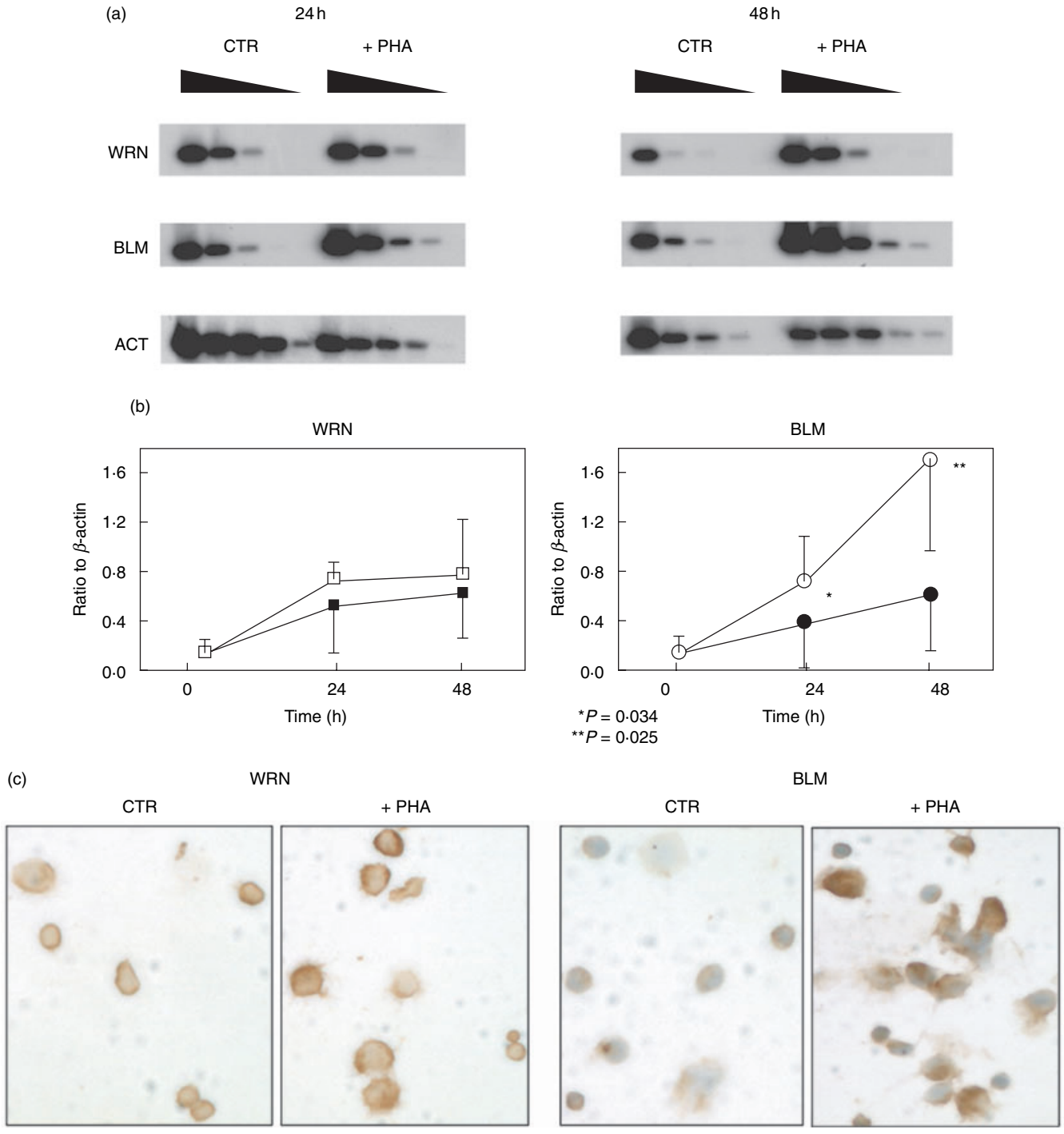

Fig. 1. Effect of PHA activation on the expression of WNR and BLM genes in normal PBMC. (a) Steady state levels of WRN, BLM and β-actin mRNA in PBMC exposed or not to PHA for 24 and 48 h. Total RNA has been retrotranscribed and amplified with selected primers in semiquantitative RT-PCR. Serial 1 : 10 dilutions of cDNA, starting from a dilution corresponding to 20 000 cells were amplified. The amplicons were revealed by Southern blotting using specific radiolabelled probes. (b) Relative levels of mRNA for WRN and BLM in PBMC after different times of induction with PHA. Quantitative data were obtained as described in the text and expressed as a ratio to β-actin mRNA. Mean values and standard deviations of seven different experiment are shown. Empty squares and circles represent the time-course of PHA-activated PBMC for WRN and BLM, respectively. Filled squares and circles represent the time-course of untreated PBMC for WRN and BLM, respectively. (c) Immunocytochemical staining of PBMC stimulated or not for 48 h with PHA. Polyclonal IgG raised in rabbits anti-BLM and anti-WRN were used as primary antibodies, as described in Materials and methods. The immunoreaction was revealed using DAB as chromogen substrates. Cells were counterstained in Mayer's acid haemalum.

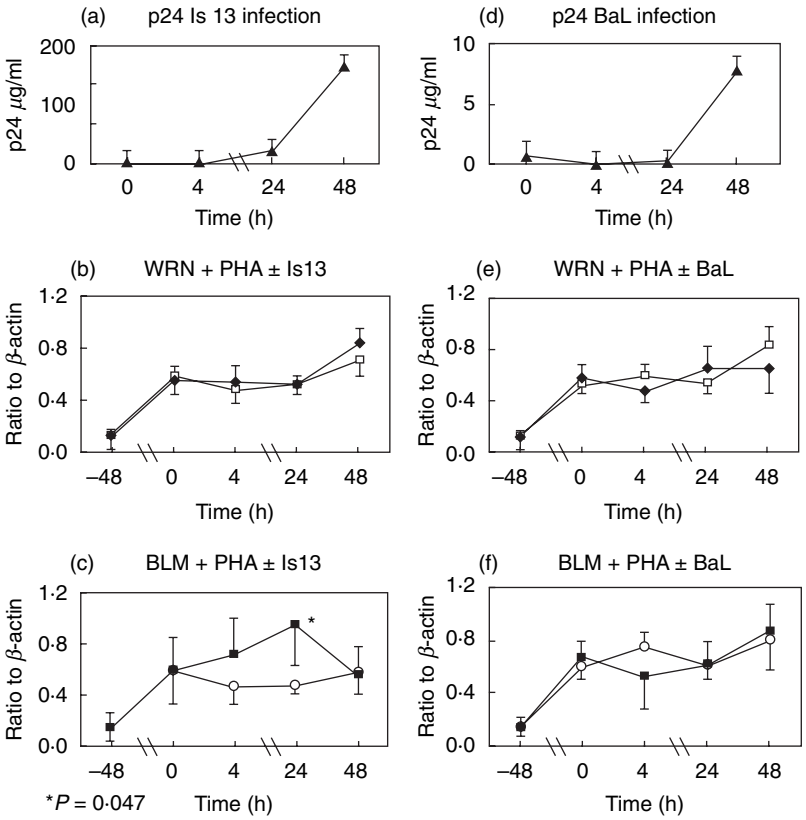

Fig. 2. Effect of HIV-1 infection on the expression of WNR and BLM genes in PHA-activated PBMC. (a,d) p24 antigen production in PHA-stimulated PBMC infected with the X4 lymphotropic HIV-1 primary isolate Is13 and the R5 macrophage-tropic strain BaL, respectively. (b,e) WNR expression, PBMC infected with X4 and R5 strains, respectively. (c,f) BLM expression, PBMC infected with X4 and R5 strains, respectively. Empty symbols: uninfected PBMC. Filled symbols: HIV-1-infected PBMC. Relative levels of mRNA for WRN and BLM before PHA stimulation (–48), at the time of infection with HIV-1 (0) and after infection (4, 24, 48 h), expressed as a ratio to β-actin mRNA. After retrotranscription, serial 1 : 10 dilutions of cDNA, starting from a dilution corresponding to 20 000 cells, were amplified, revealed by Southern blotting analysis and hybridized with specific radiolabelled probes. Quantitative data were obtained as described in the Materials and methods section. Mean values and standard deviations of five different experiments are shown.

WNR and BLM mRNA superimposable on those of uninfected PBMC throughout the course of the experiment (Fig. 2e,f, respectively).

DISCUSSION

Human RecQ helicases such as WRN and BLM are known to be up-regulated sharply during the cell cycle in human B cells transformed by EBV, human fibroblasts and SV40-transformed umbilical endothelial cells [12]. Germline mutations of these genes are known to be associated with chromosome abnormalities and cancer predisposition syndromes [38,39]. These findings support the view that the levels of WRN, BLM and RecQL1 helicases are crucial to guarantee genomic stability in actively proliferating and/or transformed cells [11]. Multiple experimental evidences suggested analogies of T cell dynamics between advanced ageing and HIV infection [19–30]. Recently, it has been suggested that through a process of continuous immune activation HIV-1 infection leads to an acceleration in the ageing process of the adaptive immune system, thus contributing to immunodeficiency [31].

In this study we show, for the first time, that the expression of WNR and BLM genes is modulated differentially in normal PBMC after PHA stimulation. mRNA levels and protein staining were clearly increased for BLM, while WNR expression was not affected in PHA-stimulated PBMC. In addition, we show here that infection with a lymphocytotropic X4 strain of HIV-1, but not with a R5 strain, determined an increase of BLM mRNA at 24 h postinfection, while WRN expression was not modulated by HIV infection. According to mRNA expression, anti-BLM antibody showed more intense staining in HIV-1-infected PBMC at 24 h, while WRN protein expression was virtually identical to the control.

By estimating proviral DNA, assuming that each infected cell may contain one copy of proviral DNA, a maximum of 0·6% PBMC were infected productively at the time-point showing the stimulation of BLM expression. This percentage fails to explain the increase observed at both the mRNA (twofold) and protein levels (1·7-fold) and suggests an indirect effect of HIV infection on bystander cells, mediated possibly by unidentified cytokine(s). The HIV strains tested in our study differed in the ability to exert such an effect, confirming that different HIV-1 strains can stimulate different cytokine activation patterns. The elucidation of such points will be a subject for our future studies.

The activation of BLM gene expression after *in vitro* HIV infection indicates that HIV is able to increase further the

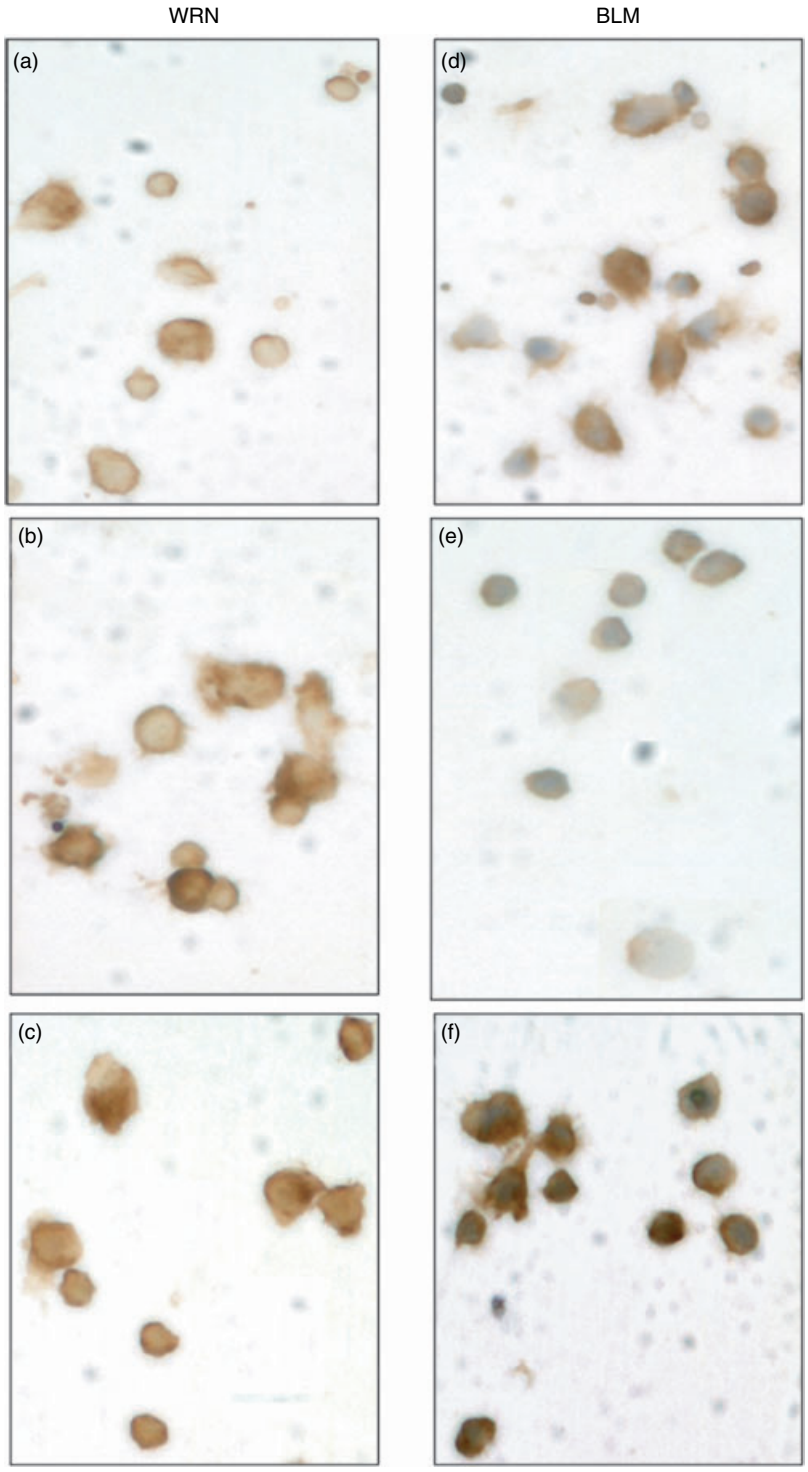

Fig. 3. Expression of WNR and BLM proteins in PHA-stimulated PBMC infected with HIV-1. PBMC were stimulated with PHA for 48 h and then infected (or not) with HIV-1 Is13. Immunostaining for WRN and BLM was performed at the time of infection and 24 h postinfection. The figure shows one representative of five independent experiments. (a,d) WRN and BLM proteins expression in PBMC at the time of infection, i.e. at 48 h of PHA stimulation. (b,e) WRN and BLM proteins expression 24 h later without infection. (c,f) WRN and BLM proteins expression in HIV-infected PBMC, 24 h postinfection.

expression of this gene in cells that are already undergoing activated BLM gene expression due to PHA stimulation. Although both WRN and BLM helicases are involved in DNA metabolic processes such as replication, recombination and/or repair, it is interesting to note their differential expression. In this respect, it should be noted that in a cultured lymphoblastoid T cell line (C8166) WNR and BLM mRNA levels are high in the absence of exogenous stimulation, and in these cells a further increase is not

observed after infection with HIV-1 (our unpublished data). The significance of BLM up-modulation is, at present, unknown, but the finding that a much lower proportion of cells harbour proviral DNA compared to the proportion of cells over-expressing BLM suggests an indirect and specific effect mediated by HIV-1.

Activation of the BLM gene in infected PBMC, shown here for the first time, suggests that premature ageing could be a further immunopathogenic mechanism involved in HIV-induced immunodeficiency, and may lead to possible new candidate targets for innovative therapeutic intervention. The analysis of BLM and WNR genes expression in PBMC from chronically HIV-1 infected patients is currently under evaluation to compare the *in-vivo* situation with that observed *in vitro*, and to establish further the relevance of our findings.

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