Interleukin 15-Mediated Induction of Cytotoxic Effector Cells Capable of Eliminating Epstein-Barr Virus-Transformed/Immortalized Lymphocytes in Culture

Ehsan Sharif-Askari, Lama M. Fawaz, Phay Tran, Ali Ahmad, José Menezes

Background: Interleukin 15 (IL-15) activates cytotoxic lymphocytes and drives the expansion of memory T cells. Its role in immune control of virus-transformed cells and other tumor cells remains to be elucidated. We investigated the role of IL-15 in controlling Epstein-Barr virus (EBV)-transformed/immortalized lymphocytes in culture. EBV is a highly potent lymphocyte-transforming and opportunistic oncogenic herpesvirus associated with several human tumors. Methods: Peripheral blood mononuclear cells (PBMCs) from healthy donors were infected with EBV and cultured with either IL-15 or IL-15 plus anti-IL-15 antibodies for 3–4 weeks. We monitored EBV-induced transformation by assessing the clearly visible cell clusters by microscopy and analyzing the expression of EBV-encoded latent membrane oncoprotein-1 (LMP-1) and the EBV nuclear antigen (EBNA) complex by immunoblotting and immunofluorescence techniques, respectively. We depleted EBV-infected cultures of PBMCs of specific effector cell populations to investigate the effector cells involved in mediating IL-15 effect. Results: The presence of IL-15 resulted in the complete elimination of EBV-transformed cells in PBMC cultures. Western blot and immunofluorescence analyses performed 3–4 weeks after infection showed no detectable levels of LMP-1 and EBNA in IL-15-treated EBV-infected cultures, whereas IL-15-untreated EBV-infected cultures and IL-15/anti-IL-15-treated cultures expressed both proteins. IL-15 mediated its anti-EBV effect through early and late response mechanisms, i.e., by first activating natural killer (NK) cells and subsequently inducing cytolytic NK-T cells. The presence of anti-IL-15 neutralizing antibodies abrogated IL-15’s effect on both mechanisms. Conclusion: In vitro, IL-15 mediated complete elimination of EBV-infected/transformed lymphocytes via successive activation of NK and NK-T cytotoxic effectors. If these in vitro findings reflect in vivo mechanisms, then IL-15 might be considered for cytokine-based immunotherapy in patients with EBV-associated lymphoproliferative disorders/malignancies.

The Epstein-Barr virus (EBV), a ubiquitous human lymphotropic herpesvirus (1), has been the subject of extensive studies particularly because of its association with various human lymphoproliferative disorders and malignancies. EBV is the etiologic agent of infectious mononucleosis (IM) (2), a self-limiting lymphoproliferative illness, and has been linked to a growing list of human malignancies, including endemic Burkitt’s lymphoma (3), Hodgkin’s disease (HD) (4), immunoblastic lymphoma in immunosuppressed individuals (5), undifferentiated nasopharyngeal carcinoma (6), various T-cell lymphomas (7,8), salivary gland tumors (9), and acquired immunodeficiency-associated non-Hodgkin’s lymphoma (3,10). After primary infection, EBV persists for life in B cells mainly in a latent form (11). EBV is the only known human lymphotropic virus with the ability to induce and maintain the proliferation of infected human B lymphocytes both in vivo and in vitro (12). The EBV genome persists in infected cells either as an episome or as an integrated viral DNA, with only a limited number of EBV genes being expressed in the latently infected cell (13). The latent gene products include six nuclear antigens (EBNA-1 [EBV nuclear antigen complex], EBNA-2, EBNA-3a, EBNA-3b, EBNA-3c, and leader protein) and three latent membrane proteins (LMP-1, LMP-2A, and LMP-2B). Of these, LMP-1 and EBNA-2 proteins, for example, are known to be potent gene transactivators that can induce cellular changes consistent with cellular activation and growth (14) and play a key role in the establishment and maintenance of the transformed state (15).

Primary infection with EBV leading to IM is associated with a remarkable immune stimulation that results in the activation and expansion of lymphocytes with cytotoxic and suppressor effector functions (16,17). Although there is a strong humoral response to EBV, it is the cellular immune response that is believed to be primarily responsible for controlling EBV infection and EBV-transformed cells (18). Different effector mechanisms appear to be involved that might be regulated by cytokines released from activated cells, as well as from accessory cells after their interaction with the virus (19). It is noteworthy that it has been shown that peripheral blood lymphocytes from individuals with EBV-induced IM also display a remarkable major histocompatibility complex (MHC)-unrestricted cytotoxic activity during the acute phase of the disease (20,21).

The relative role of different cytokine-induced cytotoxic effectors in the immune control of tumor cells is not yet clear. In this context, the role of different cytokines in mediating the immune control of EBV-immortalized cells is also unknown. This is particularly true for interleukin 15 (IL-15), which can enhance the activity of cytotoxic lymphocytes. IL-15 was originally isolated from the supernatants of the simian kidney epithelial cell line CV-1/EBNA in 1994 (22). Despite a lack of amino acid sequence homology with interleukin 2 (IL-2), IL-15 has similar tertiary structure and shares many of the biologic

Affiliations of authors: Laboratory of Immunovirology, Department of Microbiology and Immunology, and Pediatric Research Center, University of Montreal and Sainte-Justine Hospital, Montreal, PQ, Canada.

Correspondence to: José Menezes, Ph.D., D.V.M., Laboratory of Immunovirology, Sainte-Justine Hospital 3175, Côte-Ste-Catherine Rd., Montreal, PQ, Canada H3T 1C5 (e-mail: jmenezes@justine.umontreal.ca).

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activities with this cytokine. These shared activities stem from the common use of the β and γ chains of the IL-2 receptor (IL-2R) by IL-15 and IL-2 for binding and inducing signal transduction (23). It has been shown that IL-15 is a T-cell chemotactant (24) and that it can inhibit apoptosis in activated T and B cells (25). In addition, IL-15 acts as a costimulator with IL-12 to facilitate the production of interferon gamma and tumor necrosis factor-α from natural killer (NK) cells (26) and promotes the induction of cytolytic effector cells, including cytotoxic T cells and lymphokine-activated killer cells (22). Although IL-15 shares biologic activities with IL-2, there are several properties of IL-15 that are distinct from those of IL-2. IL-15 uses a distinct α chain (IL-15Rα) other than the α chain of the IL-2R (27). In addition, while IL-2 is selectively expressed in activated T cells, IL-15 messenger RNA has been found constitutively expressed in several human tissues (22). This difference in the expression pattern between IL-2 and IL-15 suggests varied in vivo roles for each of these cytokines. Recently, it was shown that IL-15 drives the expansion of CD8+ memory T cells in vivo, while this effect is counterbalanced by IL-2 (28).

Previous reports from our laboratory (29–31) have demonstrated that exposure of human peripheral blood mononuclear cells (PBMCs) to different, unrelated viruses including EBV results in in immediate enhancement of IL-15 gene expression and in IL-15 secretion by infected PBMCs. More important, all of these studies demonstrated the ability of secreted IL-15 to enhance the cytotoxic activity of NK cells immediately after viral infection, thus suggesting an important role for this cytokine in antiviral innate immune response and elimination of virus-infected cells. It was, therefore, of interest to determine whether IL-15 can also generate cytotoxic effectors capable of controlling EBV-transformed/immortalized cells.

Here, we provide evidence demonstrating that the presence of recombinant human IL-15 in EBV-infected human PMBC cultures indeed results in complete elimination of EBV-immortalized cells by IL-15-activated NK and NK-T effectors. The results of this study can be highly pertinent with regard to new immunotherapeutic approaches, particularly against EBV-associated lymphoproliferative disease and possibly also against different virus-associated tumors.

**Materials and Methods**

**PBMC isolation and cell purification.** For investigating the effect of IL-15 on the immune control of EBV-transformed cells, we collected peripheral blood from eight healthy donors, seven of whom were EBV seropositive and one of whom was EBV seronegative. Blood samples were obtained from each donor after giving informed written consent and following approval of this research by this research center’s Ethics Committee. The EBV-seropositive and EBV-seronegative status of these donors was defined by the presence or absence, respectively, of antibodies to EBV capsid antigen and to EBNA by use of immunofluorescence assay (see below). PBMCs were isolated by centrifugation (400g at 20°C for 25 minutes) of blood on a Ficoll–Hypaque (Pharmacia Amersham, Biotech AB, Uppsala, Sweden) gradient by the standard procedure (29). Cells were resuspended at a concentration of 1 × 10⁶ cells/mL in complete medium composed of RPMI-1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) containing 100 IU/mL penicillin, 20 µg/mL streptomycin, 1.0 µg/mL gentamycin, and 1.0% glutamine. B cells were recovered from PBMCs by negative immunoselection by use of a commercial kit (Stem Cell Purification System; Stem Cell Technologies, Vancouver, BC, Canada), according to the manufacturer’s instructions. Briefly, PBMCs (5 × 10⁶/mL) were incubated for 30 minutes on ice with the antibody cocktail (100 µL/mL) for the enrichment of human B cells, after which the magnetic colloid (60 µL/mL) was added for an additional 30 minutes. Cells were then loaded into purification column, and the purified cells were collected in the run through the column. A similar procedure was used to isolate NK cells [CD8(-)/CD56(+)], NK-T cells [CD8(+)/CD56(+)], and CD8+ T cells [CD8(+)/CD56(-)] from IL-15-treated PBMCs. The purity of the cell populations thus obtained was greater than 95% as determined by flow cytometry analyses.

**Cell lines and reagents.** The B95-8, K562, BJA-B, and EBV-immortalized lymphoblastoid cell line (LCL) cell lines used were cultured at 37°C in the presence of 5% CO₂ in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and antibiotics as described previously (32). Human recombinant IL-15 and monoclonal antibodies (MAbs) to human IL-15 (M110 and M112) were a gift from the Immunix Corporation (Seattle, WA). IL-15 was used at a concentration of 50 ng/mL, and anti-IL-15 was used at a concentration of 10 µg/mL throughout this study. These concentrations were determined on the basis of our previous studies (29–31). The effectiveness of IL-15 neutralization by anti-IL-15 MAb was evaluated by its ability to inhibit IL-15-induced NK cell activity as described previously (29,31). Anti-LMP-1 MAb (S12 MAb) was provided by E. Kieff (Harvard University, Boston, MA).

**Preparation of EBV, infection of cells, and cytokine treatment.** EBV was obtained from cell-free supernatants of EBV-producing B95–8 cell line as described previously (33) but without the addition of phorbol myristate acetate. The viral preparation used had a titer of 10⁹ EBNA-inducing units/mL as determined by use of BJA-B cells as described previously (33). In preliminary experiments, the addition of 100 µL of this viral preparation (i.e., 10⁴ EBNA-inducing units) to PBMC cultures (10⁶ cells/tube) obtained from different blood samples invariably resulted in the growth of EBV-immortalized cells, which was confirmed by established criteria (33). For EBV infection and cytokine treatment, PBMCs (1 × 10⁶ cells) were incubated with 10⁴ EBNA-inducing units of the viral suspension or mock infected (i.e., with virus-free supernatant) for 60 minutes at 37°C in 5% CO₂. After extensive washing with complete medium, EBV- and mock-infected cells were resuspended in 1 mL of complete medium in the presence or absence of IL-15 (50 ng/mL), anti-IL-15 (10 µg/mL), or IL-15 plus anti-IL-15. The cells were then cultured in microplate wells (2 × 10⁵ cells per 200 µL/well) and were refed twice weekly with complete medium supplemented as above. We monitored the growth of EBV-transformed cells with clearly visible cell clusters by microscopy, as well as by the expression of EBV-encoded LMP-1 and EBNA complex by immunoblotting and immunofluorescence techniques, respectively.

**Cell proliferation assay.** We measured proliferation 72 hours after the indicated treatment by adding 1 µCi of [³H]thymidine 6 hours before the cells were harvested. Cells were then harvested on glass-fiber filter paper, and the incorporation of radioactivity was determined by use of a liquid scintillation counter.

**Cell depletion.** PBMCs prepared as described above were incubated with anti-CD3 or anti-CD16 MAbs for 60 minutes on ice. Cells were then washed twice and treated with rabbit complement for 60 minutes at 37°C. The cell depletion procedure was repeated (twice) to maximize cell purity. The efficiency of depletion was assessed by fluorescence-activated cell sorter analysis of viable cells and was found to be greater than 95%.

**Cell cytotoxicity assay.** Cytotoxicity assay was performed by use of a standard ⁵¹Cr-release assay as described previously (31). Each experiment was carried out in triplicate, and the results are presented as the mean ± 95% confidence intervals (CIs) of three independent determinations.

**Western blot analyses.** Western blot analyses were performed by use of standard procedures (29). S12 Mab (1:2000) specific for LMP-1 protein and goat anti-mouse immunoglobulin G coupled to horseradish peroxidase were used in immunoblotting assay. The immuneactive bands were detected by use of enhanced chemiluminescence reagents (Du Pont NEN, Boston, MA) on x-ray film.

**Indirect immunofluorescence.** EBNA detection was carried out by use of the anticomplement immunofluorescence test as described by Reedman and Klein (34). Stained preparations were examined under a fluorescence microscope.

**Statistical analysis.** The results from triplicate wells of all in vitro cell proliferation assays or from quadruplicate wells of all in vitro cell lysis analyses were averaged and were reported as mean ± 95% CIs. The 95% CI was calculated as 1.96s/√n, where s = the standard deviation of data and n = the number of tests. Differences were analyzed for significance by Student’s t test, and a P value of less than .05 was considered to be statistically significant.
RESULTS

Effect of IL-15 on Growth of EBV-Immortalized Cells In Vitro

For the investigation of the effect of IL-15 on the growth of EBV-immortalized cells in vitro, PBMCs from seven EBV-seropositive individuals and one EBV-seronegative healthy individual were infected with the use of the lymphocyte-transforming EBV strain B95–8 and incubated in the presence or absence of IL-15 and/or anti-IL-15 antibodies for 21 days. Microscopic analyses showed that, while PBMCs that were incubated without viral infection for 21 days did not show any sign of EBV-induced transformation (Fig. 1, A), EBV infection of PBMCs in vitro resulted in EBV-induced transformation with clearly visible cell clusters (Fig. 1, B). On the other hand, incubation of EBV-infected PBMCs in the presence of 50 ng/mL of IL-15 resulted in the inhibition of EBV-induced transformation and visible cell clusters through 21 days after infection (Fig. 1, C). Simultaneous addition of IL-15 and a MAb specific to IL-15 and visible cell clusters through 21 days after infection (Fig. 1, D), thus illustrating the specificity of IL-15’s effect in the control of the growth of EBV-infected/transformed cells.

Immunofluorescence analysis for detecting EBNA expression in these PBMC cultures, with the use of a reference serum from an EBV-seropositive donor, supported our microscopic results. As shown in Fig. 1, while PBMCs incubated without viral infection for 21 days did not show any sign of EBV-induced transformation (Fig. 1, E), EBV infection of PBMCs with EBV resulted in more than 90% EBNA positivity 21 days after infection (Fig. 1, F). However, EBV-infected PBMCs incubated for a similar period of time in the presence of IL-15 showed 0% EBNA positivity (Fig. 1, G). Incubation of EBV-infected PBMCs in the presence of neutralized IL-15 resulted in greater than or equal to 80% EBNA positivity (Fig. 1, H). Identical results were obtained in all experiments carried out by use of PBMCs from eight (seven EBV seropositive and one EBV seronegative) different individuals (data not shown). These results confirm that IL-15 mediates the control of the growth of EBV-transformed cells in PBMC cultures. It should be noted that, since the results of our preliminary experiments (not shown) and the present study did show consistently that the EBV-seropositive or EBV-seronegative status of the donor played no role in IL-15-induced anti-EBV effect in our assay system, the details of experimental evidence shown are from experiments using PBMCs from the same EBV-seronegative healthy donor (unless otherwise specified).

LMP-1 is an EBV-encoded, cell-transforming protein expressed in EBV-immortalized lymphocytes (15). Immunoblotting analysis was carried out to determine LMP-1 protein expression in cultures from EBV-seronegative and EBV-seropositive donors 21 days after infection in the presence or absence of IL-15. As shown in Fig. 2 (lanes 1–6), LMP-1 protein was detected in EBV-infected cells grown in the absence of IL-15 (lane 4) but was undetectable when EBV-infected cells were cultured in the presence of IL-15 for 21 days (lane 5). Moreover, addition of anti-IL-15 antibodies to EBV-infected PBMCs cultured in the presence of IL-15 resulted in the detection of LMP-1 (lane 6). No LMP-1 band was detected from PBMCs cultured without any (i.e., virus or IL-15) treatment (lane 3). The EBV-negative cell line K562 (lane 1) and EBV-positive cell line LCL (lane 2) were used as negative and positive controls, respectively. Taken together, these data clearly demonstrate that the presence of IL-15 resulted in the elimination of EBV-transformed cells in PBMC cultures.

Effect of IL-15 on EBV-Immortalized PBMCs

To determine the effect of IL-15 treatment on EBV-transformed cells, we added IL-15 to cultures at later stages of infection after evidence of EBV-induced transformation. PBMCs from an EBV-seronegative individual were either infected with EBV or mock infected, as described in the “Materials and Methods” section. Two weeks later, when EBV-induced transformation of B lymphocytes was confirmed by microscopic observation of cellular growth and EBNA antigen

![Image](https://academic.oup.com/jnci/article-fig/93/22/1724/2519601)

Fig. 1. Effect of interleukin 15 (IL-15) on Epstein-Barr virus (EBV)-induced transformation. Freshly isolated peripheral blood mononuclear cells (PBMCs) from a healthy EBV-seronegative donor were obtained as described in “Materials and Methods” section. Cells (1 × 10^6) were infected with the B95–8 EBV strain and cultured in 96-well plates (2 × 10^5 cells per 200 μL/well) in the presence or absence of IL-15 (50 ng/mL) or IL-15 plus anti-IL-15. Original magnification ×100 for upper panels and ×400 for lower panels. It should be noted here that, since the results of our preliminary experiments (not shown) and the present study consistently showed that EBV-seropositive or EBV-seronegative status of the donor played no role in IL-15-induced anti-EBV effect in our assay system, the details of the experimental evidence shown in all of the figures (unless otherwise specified) are from experiments using PBMCs from the same EBV-seronegative healthy donor. MAb = monoclonal antibody.
expression by immunofluorescence (i.e., with >60% EBNA-positive cells in EBV-infected cultures), cells were cultured in the presence or absence of IL-15 for an additional 10-day period. The effect of IL-15 on the growth of EBV-transformed cells in these cultures was then determined by detecting EBNA expression. As shown in Fig. 3, the presence of IL-15 for this additional 10-day period resulted in the elimination of EBV-infected PBMC cultures. In contradistinction, EBV-infected PBMCs incubated in the absence of IL-15 showed 93% or more EBNA positivity when cultured for a similar period of time. Mock-infected PBMCs cultured in the presence or absence of IL-15 showed 0% EBNA positivity after 25 days of incubation. These results clearly show that IL-15 is effective in controlling the growth of EBV-immortalized cells, whether added initially at the time of viral infection or after viral transformation.

Effect of IL-15 on EBNA Expression in B95–8 Cell Line, LCL Proliferation, and Purified Primary B Lymphocytes

To determine if IL-15 has any direct inhibitory effect on EBNA synthesis, we cultured cells of the EBV-producer B95–8 cell line, which contain multiple copies of the EBV genome, for 10 days in the presence or absence of IL-15. Immunofluorescence analysis indicated no substantial changes in the percentage of EBNA-expressing cells between the two culture conditions (83.0% [95% CI = 79.0% to 86.9%] versus 90.2% [95% CI = 83.4% to 97.1%]). Similar results were obtained with a cell line derived after lymphocyte transformation by EBV (LCL) in vitro (91.8% [95% CI = 85.8% to 97.7%] versus 86.2% [95% CI = 81.5% to 91.0%]). In addition, the presence of IL-15 had no notable effect on the viability and growth of B95–8 cells and LCL, as was determined by trypan blue dye exclusion and cell proliferation assays, respectively (data not shown).

Since B lymphocytes are the targets for EBV-induced immortalization in PBMC cultures (33), we further examined whether IL-15 exerts a direct inhibitory effect on the proliferation of freshly isolated B cells infected with EBV. B cells were purified from freshly isolated PBMC from EBV-seronegative and EBV-seropositive individuals by use of negative selection procedure as described in the “Materials and Methods” section. Purified B cells were then infected with EBV, cultured in the presence or absence of IL-15 and/or anti-IL-15 antibodies, and examined microscopically for the appearance of EBV-induced transformed cell clusters. Microscopic examination and EBNA expression 15 days after infection (data not shown) confirmed the transformed status of EBV-infected B cells in these cultures. IL-15 treatment for 15 days did not inhibit cell proliferation of purified EBV-infected/transformed B lymphocytes as compared with the cells cultured without IL-15 (Fig. 4). In fact, IL-15 stimulated the proliferation of EBV-infected B lymphocytes (Fig. 4). The addition of a MAb to IL-15 abrogated the proliferative effect of IL-15 treatment on B cells (P = .004). Taken together, these results suggest that IL-15 has no direct inhibitory effect on EBV-infected/transformed B cells; rather it has a stimulatory effect on their proliferation.

Effect of NK- and T-Cell Depletion on Growth of EBV-Transformed Cells

To learn about the nature of the effector cells involved in the observed inhibition of EBV-transformed cells in PBMC cultures in the presence of IL-15, we first investigated the role of the effector populations (T and NK cells) on the IL-15-mediated
Effect of interleukin 15 (IL-15) on the growth of purified Epstein-Barr virus (EBV)-infected B lymphocytes. Peripheral blood mononuclear cells from EBV-seronegative donor were first isolated, and B cells were then purified by negative selection as described in the “Materials and Methods” section. Purified B cells were then infected with EBV and cultured (2 x 10^5 cells per 200 μL/well) in the presence or absence of IL-15, anti-IL-15 monoclonal antibody (MAb), and IL-15 plus anti-IL-15 MAb. When the EBV-induced transformation in the infected B cells was confirmed by the presence of EBV nuclear antigen-positive cells, their proliferation was determined by [1^H]thymidine incorporation. The results shown represent means of triplicate wells and are expressed as counts per minute (CPM). Error bars represent 95% confidence intervals.

PBMCs were depleted of T cells (CD3+) or NK cells (CD16+) and were cultured after EBV infection in the presence or absence of IL-15 or IL-15 plus anti-IL-15 MAb. EBV-induced immortalization was assessed in these cultures by microscopic observation and monitoring of the presence of EBNA-expressing cells as determined by immunofluorescence staining at different time points (see below). As was consistently observed, IL-15 treatment of unfractonated, EBV-infected PBMCs resulted in the complete elimination of EBV-transformed cells as compared with EBV-infected PBMCs without IL-15 treatment (Table 1).

EBV infection of CD16-depleted PBMCs resulted in rapid transformation in 60% of cultured cells by day 14 (as compared with the unfractonated PBMCs) as measured by cellular growth (Table 1, A). Remarkably, the addition of IL-15 to the CD16-depleted, EBV-infected PBMCs resulted in the appearance of EBV-transformed cells by day 14 in 60% of the cultures; however, cellular growth was subsequently reduced to 30% by day 18 and to 0% by day 21 after infection in these cultures. The addition of the IL-15 neutralizing antibody (anti-IL-15) to the CD16-depleted, EBV-infected cell cultures resulted in the appearance of cellular growth by day 14 that persisted in all wells throughout the experiment (day 21), as confirmed by EBNA staining. These results suggest that NK effectors, activated in the presence of IL-15, play a crucial role in controlling EBV-infected cells in the early phase after infection; however, NK cells were not the sole population that controlled the EBV-infected/transformed cells in the presence of IL-15. Indeed, depletion of CD3+ T cells from PBMCs before EBV infection led to the appearance of EBV-transformed cells at a slower rate (day 18) as compared with that seen with CD16-depleted cultures (day 14) (Table 1, A). However, incubating CD3-depleted, EBV-infected PBMCs in the presence of IL-15 resulted in the growth of EBV-transformed cells by day 18 that remained positive for EBNA until the end of the experiment. Neutralizing anti-IL-15 antibody in these cultures (i.e., CD3-depleted, EBV-infected PBMCs in the presence of IL-15 and a MAb to IL-15) further enhanced the EBV-induced cell growth process to levels comparable to those seen with CD16-depleted, EBV-infected PBMCs cultures. The EBV serologic status of the donor did not play a role in the IL-15-mediated anti-EBV effect (Table 1, B). Taken together, results from CD16-depleted and CD3-depleted cultures strongly suggested that the IL-15-mediated inhibitory effect is exerted by NK cells in the early-phase post-EBV infection and by a T-cell population thereafter.

Table 1. Effect of natural killer (NK) and T-cell depletion on Epstein-Barr virus (EBV)-induced immortalization in the presence or absence of interleukin 15 (IL-15)

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*The results shown here were obtained by use of peripheral blood mononuclear cells (P) from EBV-seronegative (Part A) and EBV-seropositive (Part B) donors. Each determination was carried out by use of 10 replicate culture wells (2 x 10^5 cells per 200 μL/well). + represents cultures where EBV-induced transformation was observed in all wells (100%) unless otherwise specified in parentheses; – represents cultures where no EBV-induced transformation was observed. EBV-induced transformation was assessed by microscopic examination and EBV nuclear antigen staining was as described in the “Materials and Methods” section. The numbers given in parentheses represent percent of wells showing transformed cultures.

Effect of IL-15-Induced NK-T Cells on Growth of EBV-Infected/Transformed Cells

The data that we described above clearly indicated that both NK and T cells were involved in the IL-15-induced inhibitory activity against EBV-transformed/imortalized cells. Previous studies (24,31) have shown that IL-15 is a potent activator of both NK and T cells. During the present experiments, we also found that IL-15 treatment of PBMCs resulted in a statistically significant induction of lymphocyte proliferation that was com-
pletely abrogated when we used IL-15 neutralized with anti-IL-15 antibody (Fig. 5, A) \( (P = .0001) \). Therefore, we thought it important to determine the phenotype as well as the cytolytic activity of the proliferating cells. For this purpose, IL-15-treated PBMCs were first analyzed by flow cytometry by use of different fluorescein isothiocyanate-conjugated MAbs against NK- and T-cell surface markers. Data from flow cytometry analyses revealed that, while IL-15 had a notable proliferative effect on CD8+ T cells (Fig. 5, B), no such proliferative effect was observed on CD4+ T lymphocytes (Fig. 5, C) as compared with untreated cells. Furthermore, two-color flow cytometry analysis of IL-15-treated PBMCs clearly showed that, while IL-15 specifically and considerably induced proliferation of CD8(+)+CD56(+) NK-T-cell population (Fig. 6, A), it did not induce CD3(-)+CD16(+) NK cell proliferation (Fig. 6, B). For the direct determination of the cytolytic capacity of these CD8(+)+CD56(+) NK-T lymphocytes, this cell population was purified from IL-15-treated PBMCs by negative selection as described in the “Materials and Methods” section. Purified NK-T lymphocytes were then incubated with target cells and tested for their ability to lyse these cells. Our results clearly reveal that these IL-15-activated, purified CD8(+)+CD56(+) NK-T lymphocytes are powerful killers at different effector-to-target ratios (Fig. 7, A). Two other populations, including CD8(+)+CD56(-) T cells and CD8(-)+CD56(+) NK cells were also purified from similar treatment and tested for their cytolytic activity. The data obtained revealed that CD8(-)+CD56(+) NK cells exhibited relatively low cytolytic activity against K562 target cells (at effector-to-target ratio of 40:1) (Fig. 7, B). This cytolytic activity of the NK cells after 21 days of IL-15 treatment did not differ substantially from that observed 24 hours after IL-15 treatment (28.5% [95% CI = 26.6% to 30.4%] versus 33.9% [95% CI = 31.7% to 35.8%], respectively). Furthermore, the number of CD8(-)+CD56(+) cells obtained from IL-15-treated PBMCs was considerably lower than that of CD8(+)+CD56(+) NK-T cells isolated from these PBMCs (0.5 × 10⁹ versus 20 × 10⁶, respectively). On the other hand, purified CD8(+)+CD56(-) T lymphocytes from IL-15-treated PBMCs had no remarkable killing activity against K562 target cells (Fig. 7, B) as compared with both CD8(-)+CD56(+) NK or CD8(+)+CD56(+) NK-T cells. Taken together, these results clearly indicate that IL-15 treatment not only sustained the NK cytolytic activity but also induced the expansion of highly cytolytic CD8(+)+CD56(+) NK-T lymphocytes.

**DISCUSSION**

We demonstrated here that addition of IL-15 to EBV-infected PBMC cultures results in complete abrogation of the growth of EBV-transformed/immortalized cells. Indeed, immunofluorescence analysis for EBNA expression and immunoblotting for LMP-1 did not reveal any detectable levels of these antigens after treatment of EBV-infected PBMC cultures with IL-15 for 21 days. Furthermore, IL-15 had no effect on EBNA synthesis or growth of purified B lymphocytes or of established EBV-immortalized B cell lines in vitro, suggesting that the IL-15-mediated effect on EBV-transformed/immortalized cells is not a direct effect of this cytokine on cell killing or on cell growth arrest of the target cell population. To the contrary, IL-15 treatment enhanced the proliferation of EBV-transformed B cells. These findings are consistent with previous reports, whereby IL-15 costimulated the proliferation and differentiation of purified tonsillar B cells preactivated with either anti-immuno-
globulin M or phorbol ester (35). Similarly, Trentin et al. (36) demonstrated the ability of IL-15 to trigger the proliferation of freshly isolated leukemic B cells but not normal resting B lymphocytes. Taken together, our results clearly demonstrate that IL-15 does not have a direct antiviral effect, but rather it induces cytotoxic effectors that control EBV-immortalized cells. This IL-15-mediated control was independent from the EBV serologic status of PBMC donors.

NK and nonspecific T cells were suggested to play an essential role in host defenses aimed at controlling viral spread during the acute phase of infection through the induction of death of virus-infected cells (17). Considering that the abrogation of EBV-transformed/immortalized cells was not due to the direct effect of IL-15 on these cells, we sought to determine the effector cells involved in the elimination of the EBV-infected targets in PBMC cultures. Infection of unfractionated PBMCs with EBV resulted in remarkable growth of immortalized cells after 18 days of infection. However, the growth of EBV-transformed cells was abrogated completely when EBV-infected PBMCs were cultured in the presence of IL-15. Depletion of CD16-positive NK cells clearly revealed the important role of these cytolytic cells immediately after viral infection, since their absence in PBMCs before EBV infection led to accelerated growth of virus-immortalized cells. This is consistent with recently published reports (37,38), in which IL-15 was shown to reduce herpesvirus infection in vitro through the activation of NK cells in short-term experiments. This result together with our present results, showing an incomplete control of EBV-infected transformed cells at the early stages after infection, strongly indicates that additional effector cells may be required for a complete IL-15-mediated control of cells infected by EBV (and possibly also by other herpesviruses).
Results from the addition of IL-15 to PBMC cultures strongly suggested the ability of IL-15 to exert a stastically significant proliferative effect in cell cultures (P<.001). Phenotypic analysis revealed that IL-15, while having no remarkable effect on the growth of CD4+CD56+ NK-T lymphocytes during 21 days of culture. Results of cytotoxic assays strongly suggest that the expansion of these highly cytolytic NK-T effector cells in the presence of IL-15 could represent the second step in the induction process of antiviral effector mechanisms by this cytokine, thus ensuring an appropriate and extended innate immune response. Thus, these mechanisms would involve, first, an enhancement of NK cytolytic activity in the early stage of infection and, second, an induction of highly cytotoxic CD8+CD56+ NK-T cells in the later stage of infection. Such NK-T cells have been shown previously to be potent and MHC-unrestricted killer cells that induce cell death in their targets mainly through granule exocytosis (39). Furthermore, IL-15-treated PBMCs also contained a very small number of CD8−CD56+ NK cells that maintained their basal level cytolytic activity in the presence of IL-15 during the incubation time. This result is in agreement with previously published reports in which a picomolar amount of IL-15 was demonstrated to sustain the survival of resting human NK cells for several days (40). In any event, although the precise mechanism of the NK and NK-T-cell effects leading to deletion of EBV-transformed cells is not clear, there is no reason to believe that it is not due to their cytolytic activity against these EBV-positive cells. Further studies will be required to address this issue.

Several lines of evidence validate the important role of IL-15 in the maintenance and expansion of NK-T cells in vivo. Genetic evidence from IL-15−/− mice strongly supports the crucial role of IL-15 in the development of NK-T cells (41). IL-15−/− mice displayed a pronounced reduction in the number of thymic and peripheral NK-T cells as well as a reduced number of splenic memory CD8+ T cells, whereas conventional T-cell numbers were not affected. More important, these mice had an increase in memory CD8+ T cells, whereas conventional T-cell numbers.

T-cell homeostasis has also been emphasized by a recent study (28) on its key role in the survival and expansion of memory CD8+ T cells.

In conclusion, the present in vitro data clearly show that IL-15 can play a crucial role in the control of EBV-transformed/immortalized cells and that both NK and NK-T lymphocytes are important effectors in this control: NK cells in the early phase, i.e., immediately after EBV infection, and NK-T lymphocytes in later stages after infection, i.e., from about the second to the third week after infection. If these in vitro results are to reflect the human host’s antiviral effector mechanisms against EBV-infected/transformed cells in vivo, one might then be tempted to consider the possibility that IL-15-mediated immunotherapy may be of help in patients with EBV-induced lymphoproliferative states and malignancies as well as in patients with chronic active EBV infection. Of interest, IL-15 use seems to present advantages over the use of IL-2, which has been shown to pose a major toxicity problem causing a vascular leak syndrome that can ultimately lead to organ failure when administered in immunotherapy (44). Indeed, when IL-15 and IL-2 were used at a comparable dose in a mouse model of metastatic tumor, IL-15 was found to cause a threefold less pulmonary vascular leak than IL-2 (24). More important, IL-15’s unique role in the control of virus-infected/transformed cells through the expansion of a cytotoxic NK-T-cell population would constitute an incentive for experimenting with the use of this cytokine in immunotherapeutic approaches, in particular for the treatment of EBV-induced lymphoproliferative diseases. The fact that IL-15 also contributes to the expansion of memory CD8+ T cells is also highly pertinent in this regard. Furthermore, whether IL-15-induced cytotoxic effectors can play a protective role in patients with chronic active EBV (and other viral) infections remains to be addressed.

References


NOTES

E. Sharif-Akari and L. M. Fawaz contributed equally to this work.

Present address: E. Sharif-Akari, Department of Microbiology and Immunology, McGill University, Montreal, PQ, Canada.

Present address: L. M. Fawaz, Department of Medicine, Division of Experimental Medicine, McGill University, Montreal, PQ, Canada.

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