IL-21 imposes a type II EBV gene expression on type III and type I B cells by the repression of C- and activation of LMP-1-promoter

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Epstein–Barr virus (EBV) is associated with a variety of human tumors. Although the EBV-infected normal B cells *in vitro* and the EBV-carrying B cell lymphomas in immunodeficient patients express the full set of latent proteins (type III latency), the majority of EBV-associated malignancies express the restricted type I (EBNA-1 only) or type II (EBNA-1 and LMPs) viral program. The mechanisms responsible for these different latent viral gene expression patterns are only partially known. IL-21 is a potent B cell activator and plasma cell differentiation-inducer cytokine produced by CD4⁺ T cells. We studied its effect on EBV-carrying B cells. In type I Burkitt lymphoma (BL) cell lines and in the conditional lymphoblastoid cell line (LCL) ER/EB2-5, IL-21 potently activated STAT3 and induced the expression of LMP-1, but not EBNA-2. The IL-21-treated type I Jijoye M13 BL line ceased to proliferate, and this was paralleled by the induction of IRF4 and the down-regulation of BCL6 expression. In the type III LCLs and BL lines, IL-21 repressed the C-promoter-derived and LMP-2A mRNAs, whereas it up-regulated the expression of LMP-1 mRNAs. The IL-21-treated type III cells underwent plasma cell differentiation with the induction of Blimp-1, and high levels of Ig and Oct-2. IL-21 might be involved in the EBNA-2-independent expression of LMP-1 in EBV-carrying type II cells. In light of the fact that IL-21 is already in clinical trials for the treatment of multiple malignancies, the *in vivo* modulation of EBV gene expression by IL-21 might have therapeutic benefits for the EBV-carrying malignancies.

Epstein–Barr virus | IL-21 | lymphoma | STAT3

Epstein–Barr virus (EBV) is a gamma-herpes virus that has succeeded in colonizing 95% of the human population (1, 2). Generally the infection occurs in childhood and is asymptomatic; thereafter, the infected person becomes a virus carrier for their lifetime. This harmless coexistence between the virus and its host is achieved through two mechanisms: a potent immunological reaction to the virally encoded latent proteins that eliminates the type III latent cells, and a viral strategy to down-regulate the expression of latent genes and reside in a silent state in the memory B cell pool (1, 2).

The EBV genome has been detected in a variety of malignancies (1, 3). The latent gene expression seen in the EBV-carrying tumors varies according to the tissue origin of the tumors and activation/differentiation stage of the malignant cells (3). The EBV-positive Burkitt lymphoma (BL), primary effusion lymphomas, and some nasopharyngeal carcinomas (NPC) express only the EBNA-1 protein (type I latency), whereas classical Hodgkin lymphomas (cHL) (4), a part of NPCs (5), nasal NK/T lymphomas (6), peripheral T cell lymphomas (7), some diffuse large B cell lymphomas (8) and posttransplant lymphomas (PTLDs) (9, 10), and EBV-positive B cells in angioimmunoblastic T cell lymphomas (11) express EBNA-1, LMP-1, and LMP-2 (type II latency) (1, 3).

EBNA-2 and EBNA-5 (-LP) regulate the expression of LMP-1 in type III latent lymphoblastoid cell lines (LCLs) (5, 12). The molecular mechanisms responsible for the expression of LMP-1 in type II latency (EBNA-2-independent LMP-1 expression) are only

partially known. EBNA-2 is also instrumental in the activation of C- and LMP-2-promoters in type III latent cells (13). LMP-1 can be expressed from two alternative promoters: the EBNA-2-responsive ED-L1 or the promoter located in the first terminal repeat (TR-promoter) (14). Two binding sites for the STAT transcription factors were identified in the LMP-1 promoters (one in each) (15). Furthermore, STAT3, activated by IL-6, was shown to activate the TR LMP-1 promoter in epithelial cells (16, 17).

IL-21 is a cytokine homologous to IL-15, IL-2, and IL-4, and is produced by activated CD4⁺ T cells and NKT cells (18). A major source of IL-21 is follicular helper T (T_{FH}) cells found in the B cell areas of secondary lymphoid tissue (19). IL-21 signals through a heterodimeric receptor comprised of the IL-21 receptor (IL-21R) and the common gamma chain of the IL-2 receptor family. IL-21 induces human B cell activation and proliferation (20, 21), Ig class switch recombination (CSR), and plasma cell (PC) differentiation of normal (21) and EBV-transformed B cells (22). Here we provide evidence for a role for IL-21 in modulating of EBV latent gene expression in several EBV-carrying B cell lines.

Results

Type I BL and *in Vitro* Converted Cell Lines. Because both IL-10 and IL-21 are potent activators of STAT3, and because we previously found that IL-10 treatment could induce the expression of LMP-1 in EBV-carrying BL and NK lymphoma cell lines (23), we tested the effect of recombinant human IL-21 in a panel of BL cell lines. The IL-21R was expressed in three type I and two type III latent Mutu BL lines tested (Fig. 1A). The type I (cl.13) and type III (cl. P79) Jijoye BL cells, and the *in vitro*-infected HL-derived cell line KMH2-EBV expressed the IL-21R (Fig. S1). IL-21 induced the phosphorylation of STAT3 at Tyr-705 in the Mutu I BL clones 59 and 148, but not cl. 216, and in two Akata EBV-converted DG75 BL sublines (Fig. 1B). Induction of LMP-1 expression in the absence of EBNA-2 paralleled the STAT3 activation (Fig. 1C and D). LMP-1 was detected already after 6 h and reached its maximum level at 24 h (Fig. 2A), whereas the expression of EBNA-1 remained unaffected. Induction of LMP-1 was not part of the lytic cycle, because the LMP-1 and early antigen (EA) stainings did not overlap (Fig. 2B). Furthermore, in line with the known inhibitory effect of LMP-1 on the entry to the lytic cycle (24, 25), the percentage of cells entering spontaneously in the lytic cycle decreased from ~5–8% to 1–2% (Fig. 2B).

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Fig. 1. Expression and functionality of the IL-21R in BL lines. (A) Expression of IgM, CD23, and IL-21R in the type I BL and type III BL lines as assessed by FACS analysis. Shaded areas represent the background fluorescence of the control Ab-stained cells; solid black lines denote the specific staining. (B) Immunoblot analysis of total cell extracts of IL-21-treated (100 ng/mL, 30 min) BL lines with phospho-STAT3 (Tyr-705)- and total STAT3-specific antibodies. (C) Expression of LMP-1, EBNA-2, and β -actin in the total cell lysates of IL-21-treated (50 ng/mL, 24 h) type I Mutu BL sublines as assessed by SDS/PAGE and immunoblotting. (D) Expression of LMP-1, EBNA-2, and β -actin in the total cell lysates of IL-21-treated (50, 5, or 0.5 ng/mL, 24 h) DG75-Akata sublines as assessed by SDS/PAGE and immunoblotting.

IL-21 induced a 167-fold increase of the LMP-1 mRNAs (originating either from the ED-L1 or the TR promoters) in the Jijoye M13 and a 181-fold increase in the Mutu I cl.59 (Fig. 2C). Using the same cDNA library to measure the expression of LMP-1 mRNAs with PCR primers located upstream of the first LMP-1 exon, which should not amplify the RNAs originating from the ED-L1 promoter but only those upstream of the ED-L1 (possibly from the TRp), the expression of these LMP-1 mRNAs was up-regulated 8-fold in the Jijoye M13 cells and 9-fold in the Mutu I cl.59 cells (Fig. 2C). IL-21 treatment did not affect the Qp-derived mRNAs but up-regulated 6-fold the LMP-2A mRNAs in the Mutu I cl.59 cells (Fig. 2C).

To provide genetic evidence that the LMP-1 induced by IL-21-induced LMP-1 is EBNA-2-independent, we infected the EBV-negative Ramos BL line with the recombinant B95-8 EBV strain in which the EBNA-2 gene is deleted (26). IL-21 induced LMP-1 in these cells as well (Fig. 2D).

The IL-21-treated Jijoye M13 cells expressed high levels of CD54 (ICAM-1), CD83, CD86, CD95 (Fas), HLA cl. I, and HLA cl. II-DQ, and down-regulated the levels of IgM and CD10 (Fig. S24). In 50–60% of the treated cells, CD83 and LMP-1 were coexpressed (Fig. S2B). IL-21 inhibited markedly the proliferation of the Jijoye M13 cells (Fig. 3A): their growth arrested in the G₀/G₁ phase of the cell cycle (Fig. 3B).

IL-21 treatment induced IRF4 with the concomitant down-regulation of BCL6 expression in the Jijoye M13 cell line but not in the Mutu I cl.59 line (Fig. 3C). Blimp-1 protein was not detected and the levels of c-myc, Oct2, and Pax5 did not change (Fig. 3C). Furthermore, the nonclassical NF- κ B pathway was

Fig. 2. Effect of IL-21 on the EBV gene expression in type I BL lines. (A) Immunoblot analysis of total cell extracts of Jijoye M13 and Mutu I cl.59 BL lines treated with 50 ng/mL IL-21 for 6, 24, or 48 h with EBNA-1, LMP-1, EBNA-2, and β -actin antibodies. (B) Double immunofluorescence staining for LMP-1 and EA of the IL-21-treated Jijoye M13 and Mutu I cl.59 BL cells (50 ng/mL, 24 h) (LMP-1 is red, EA is green). (C) Expression of total LMP-1, TR-LMP-1, LMP-2A, and QU mRNAs assessed by quantitative real-time PCR in cDNAs prepared from the Jijoye M13 and Mutu I cl.59 cells treated with 50 ng/mL IL-21 for 6 h (normalized values are shown relative to the GAPDH control). (D) Expression of LMP-1, EBNA-1, and β -actin in the total cell lysates of IL-21-treated (50 ng/mL, 24 h) Ramos-EB2KO cl.3 and cl.6 sublines as assessed by SDS/PAGE and immunoblotting. The histogram plots show the fluorescence intensity of the Ramos-EB2KO cl.3 and cl.6 measured in FL1 (solid black lines, GFP), compared to the parental, EBV-negative Ramos cells (shaded gray areas).

induced and activated (as shown by the up-regulation of p100 and its processed product, p52) in the Jijoye M13 cells but only weakly in the Mutu I cl.59 cells (Fig. 3C). Because LMP-1 is known to inhibit the proliferation of BL lines (27, 28), the antiproliferative effect of IL-21 seen only in the Jijoye M13 cells is probably due to the strong activation of the downstream signaling pathways activated by the cytokine-induced LMP-1.

Because IL-21 strongly activated STAT3 in the type I BL lines (Fig. 14) and STAT3 was shown to activate the LMP-1 TR promoter in epithelial cells (15, 16), we tested whether STAT3 alone could induce LMP-1. In two Mutu I cl.59 clones transfected with an expression vector carrying the fusion gene of STAT3 with the hormone-binding domain of the estrogen-

Fig. 3. Effect of IL-21 on the proliferation and cellular phenotype of the Jijoye M13 and Mutu I cl.59 BL cells. (A) Proliferation of the IL-21 (50 ng/mL, 3 days)-treated Jijoye M13 and Mutu I cl.59 BL cells as measured by dye-exclusion assay. Gray indicates the living cell numbers, and black indicates the dead cell number. The mean values are shown, calculated from five individual experiments. (B) Cell cycle analysis of the IL-21 (50 ng/mL)- or goat anti-human IgM antibodies (10 μ g/mL)-treated (48 h) Jijoye M13 and Mutu I cl.59 BL as measured after a 1-h incorporation of EdU. The dot histograms show the fluorescence intensity of the specific staining for the incorporated EdU (FL1, y axis) and PI (FL3, x axis). The percentage of events in the G₀/G₁ and S phases of the cell cycle are indicated in the upper right corner of the plots. (C) Immunoblot analysis of total cell extracts of IL-21-treated Jijoye M13 and Mutu I cl.59 cells with IRF4, BCL6, c-Myc, Blimp-1, Pax5, Oct-2, and NF- κ B2 (p100/p52) antibodies. The cells were treated with 50 ng/mL IL-21 for 6, 24, or 48 h before the lysates were prepared. (D) Expression of LMP-1, total STAT3, ER α , EBNA-1, and β -actin proteins in total cell extracts of empty vector (LZRS cl.1 and cl.3)- and LZRS-STAT3ER-IRES-EGFP (cl.2 and cl.3)-transfected Mutu I cl.59 BL clones treated with 1 μ M 4-OH-tamoxifen (4-HT) for 48 h.

receptor alpha (STAT3-ER) (29) 4-OH-tamoxifen (4HT) treatment induced the expression of LMP-1 protein, whereas this did not happen in two control vector-transfected clones (Fig. 3D).

Conditional LCL ER/EB2-5 Cells. The expression of EBNA-2 and LMP-1 and the proliferation of the conditional LCL ER/EB2-5 cells depends on the addition of estrogen. Upon estrogen withdrawal, the cells down-regulate EBNA-2 and LMP-1, and stop proliferating, but survive for 3–4 days (30). The addition of IL-21 in the absence of estrogen for 3 days led to a decline of EBNA-2 (Fig. S3A). However, LMP-1 expression and proliferation were maintained (Fig. S3B). The IL-21-treated cells expressed c-myc and cyclin E, and differentiated into Ig-producing, Blimp-1-positive plasmablasts (Fig. S3C).

When the ER/EB2-5 cells were starved of estrogen for 3 days and thereafter treated with IL-21 or estrogen, LMP-1 was induced (Fig. S3D) and the cells incorporated thymidine for 3 days after the addition of a single dose of IL-21 (Fig. S3E). Furthermore, cyclin E and Blimp-1 were induced, and the expression of IgM, Ig-light chain, and Oct2 was up-regulated (Fig. S3F).

LCLs and Type III BL Cell Lines. The LCLs (cord blood-derived CBM1-Ral-STO and CB-LCL-JijM13 and the tonsillar B cell-derived TB-LCL) expressed IL-21R (Fig. 4A) and activated STAT3 upon IL-21 treatment (Fig. 4B). In the IL-21-treated CBM1-Ral-STO (CBM1) cells, EBNA-1, EBNA-2, and EBNA-6 were down-regulated, and LMP-1 was either maintained or up-regulated (Fig. 4C). The down-regulation of EBNA-6 was a result of the inhibition of transcription from the EBV C promoter, as assessed by quantitative real-time PCR assays (Fig. 4D). Down-regulation was observed already between 9 and 12 h after addition of IL-21, at which time points it decreased to 63% and 41% of the untreated samples (Fig. 4D). In parallel, LMP-2A mRNAs were down-regulated (Fig. 4D), whereas LMP-1 mRNAs were up-regulated already at 3 h (Fig. 4D). The expression of Qp-derived RNAs at 2 days was unaffected but was induced 12.5-fold after 6 days of IL-21 treatment (Fig. 4E).

After 12 h of IL-21 treatment, when Cp mRNAs were already decaying, the levels of EBNA-1 and EBNA-2 proteins were still unaffected (Fig. S4). Blimp-1 was induced, Oct2 was up-

Fig. 4. Effect of IL-21 on the EBV gene expression in LCLs. (A) Expression of IgM, CD23, and IL-21R in the LCLs as assessed by FACS analysis. Shaded areas represent the background fluorescence of the control Ab-stained cells; solid black lines denote the specific staining. (B) Immunoblot analysis of total cell extracts of IL-21-treated (100 ng/mL, 30 min) LCLs with phospho-STAT3 (Tyr-705)-, total STAT3-, and actin-specific antibodies. (C) Expression of EBNA-2, EBNA-1, EBNA-6, LMP-1, and β -actin proteins in the total cell lysates of IL-21-treated (100 ng/mL, indicated times) CBM1 cells. (D) Expression of Cp-derived, LMP-2A, and LMP-1 mRNAs assessed by quantitative real-time PCR in cDNAs prepared from the CBM1 cells treated with 100 ng/mL IL-21 for 3, 6, 12, or 24 h. The normalized values are shown relative to the GAPDH control. The untreated control sample at 3 h was set to 100%. The error bars indicate $\pm$ SD. (E) Expression of QU mRNAs assessed by quantitative real-time PCR in cDNAs prepared from the CBM1 cells treated with 100 ng/mL IL-21 for 2 or 6 days. The normalized values are shown relative to the GAPDH control. The untreated control sample at 2 days was set to 100%.

regulated, Pax5 did not change, and BCL6 was not induced (Fig. S4).

In parallel with these changes, the IL-21-treated CBM1 cells differentiated toward PCs as evidenced by the induction of Blimp-1 and high Ig expression (Fig. 5A). Oct-2 and c-myc were up-regulated already after 6 h of treatment (Fig. 5A). Re-addition of IL-21 could maintain the CBM1 cells in culture for up to 10 days with the features of EBNA-2-negative, EBNA-6-negative, EBNA-1-low, and LMP-1-positive expression (Fig. 5B). Withdrawal of IL-21 after 6 days of treatment led to down-regulation of LMP-1 already in the next 24 h (Fig. 5C) and re-expression of EBNA-2 after 48–72 h (Fig. 5C).

When the CBM1 cells were exposed to IL-2, IL-4, IL-6, IL-10, IL-15, IL-21, IFN- α , or IFN- γ , only IL-21 treatment resulted in EBNA-2 down-regulation that was paralleled by the induction of Blimp-1 expression (Fig. 5D).

However, newly EBV-infected normal B cells did not respond similarly to IL-21. After 3 and 6 days of IL-21 exposure, Cp activity was not influenced (Fig. 5E), whereas the message of LMP-1 was up-regulated 2-fold (Fig. 5E).

Fig. 5. Effect of IL-21 on the cellular gene expression in LCLs and type III BL lines. (A) Expression of Blimp-1, Oct-2, total Ig, and c-myc proteins in the total cell lysates of IL-21-treated (100 ng/mL, indicated times) CBM1 cells. (B) Expression of EBNA-6, EBNA-2, EBNA-1, LMP-1, and β -actin proteins in total cell extracts of CBM1 cells treated with 100 ng/mL IL-21 for 6, 8, or 10 days. (C) Immunoblot analysis of total cell extracts of IL-21-treated CBM1 cells with LMP-1, EBNA-2, Blimp-1, and β -actin antibodies. The cells were treated with IL-21 for 6 days, after which they were washed in PBS and recultured in complete medium without IL-21 for an additional 1, 2, or 3 days. (D) Expression of EBNA-2, LMP-1, and β -actin proteins in total cell extracts of CBM1 cells treated with 1,000 units/mL IL-2 or 100 ng/mL IL-4, IL-6, IL-10, IL-15, IL-21, IFN- α , or IFN- γ for 3 days. (E) Expression of Cp-derived and LMP-2A mRNAs assessed by quantitative real-time PCR in cDNAs prepared from the B95-8-infected isolated normal B cells treated with 100 ng/mL IL-21 for 3 or 6 days. The normalized values are shown relative to the GAPDH control. The untreated control sample at 3 days was set to 100%. The error bars indicate SD. (F) Expression of EBNA-2, LMP-1, Blimp-1, c-myc, and β -actin proteins in total cell extracts of Jijoye P79, Mutu III cl.99, AG876, BL16, and BL18 cells treated with 100 ng/mL IL-21 for 3 days. (G) Expression of Cp-derived mRNAs assessed by quantitative real-time PCR in cDNAs prepared from the IL-21-treated (100 ng/mL, 12 h) type III BL lines Jijoye P79 and Mutu III cl.99 and the Nadia LCL. The normalized values are shown relative to the GAPDH control. The untreated Nadia sample was set to 100%. The error bars indicate SD.

Some of the EBV-carrying BL lines during their *in vitro* culturing spontaneously broaden the EBV latent gene expression from type I to type III latency (31). Similarly to the LCLs, IL-21 treatment down-regulated EBNA-2 in the type III BL lines, whereas LMP-1 was maintained and Blimp-1 was induced (Fig. 5F). IL-21 inhibited the expression of Cp-derived mRNAs already at 12 h in the Mutu III cl.99 and Jijoye P79 type III BL lines, and in the LCL Nadia (Fig. 5G). Furthermore, Qp was activated 2.9-fold in the Jijoye P79 cells after 6 days of IL-21 treatment.

Discussion

Being a potent activator of STAT3, IL-21 may be involved in the LMP-1 expression in EBV-carrying type II latent B cells. As we previously proposed for IL-10 (23, 32), IL-21 could be involved in the LMP-1 expression of type II normal GC B cells. T_{FH} CD4⁺ cells in the GCs of secondary lymphoid follicles are the main producers of IL-21 (19) and thereby during T–B cell interactions the expression of LMP-1 may be induced in the EBV-carrying GC B cells. The transient and CD4⁺ T cell-dependent induction of LMP-1 may secure that these GC B cells preferentially enter the memory B cell path, because these latter cells are the latent reservoir of EBV. The alternative PC differentiation leads to disruption of latency and initiation of virus replication (33, 34).

IL-21 may be involved in the EBNA-2-independent LMP-1 expression in EBV-carrying tumors as well. Two recent studies reported the expression of IL-21 and its receptor by Hodgkin-Reed/Sternberg cells (35, 36). IL-21 could be responsible for the EBNA-2-negative, LMP-1-positive viral gene expression seen also in the EBV-positive B cell proliferations in the angioimmunoblastic T cell lymphomas (11). These malignant T cells are believed to originate from the T_{FH} cells of GCs (37, 38), and therefore they probably produce IL-21. As such, IL-21 may be produced by the EBV-carrying cells or by the CD4⁺ T cells in the microenvironment.

IL-21 induced the expression of LMP-1 in the type I BL lines. In line with the known effects of LMP-1 (27, 39), the phenotype of the IL-21-treated type I BL cells changed. The Jijoye M13 cells ceased to proliferate, IRF4 was induced, and BCL6 was down-regulated. IRF4 expression was shown to be inducible through the NF- κ B pathways activated by the CD40 engagement (40) and to inhibit the expression of BCL6 by directly binding to its promoter, resulting in cell cycle arrest in BL lines (40, 41). Therefore, it is reasonable to assume that, similarly to the CD40 activation, the IL-21-induced LMP-1 was responsible for the induction of IRF4 expression and the down-regulation of BCL6 expression. Furthermore, overexpression of IRF4 was shown to inhibit the proliferation of a BL line (41).

IL-21 induces human B cell activation, proliferation, and Ig CSR (20). IL-21 is a potent inducer of Blimp-1 (21, 29), the master regulator of PC differentiation (42). The differentiation of LCLs into PC in vivo (43) or in vitro (44, 45) resulted in the down-regulation of EBNA expression, but only IL-21 treatment could maintain the expression of LMP-1 in the absence of EBNA-2 and result in the activation of Qp.

According to Konforte et al. (46), treatment of LCLs with IL-21 led to the down-regulation of EBNA-2, followed by the decrease of Cp-derived RNAs. In their experiments, both the Cp- and the LMP-2A-derived mRNAs decayed at 48 h, whereas at 24 h no differences were found. They concluded that the inactivation of Cp and LMP-2A promoter were secondary to the decrease in the EBNA-2 protein expression (46). However, our quantitative real-time PCR assays showed 37% and 59% inhibition of Cp activity already at 12 and 24 h, respectively, and a decrease in the LMP-2A message to 62% and 47%. Furthermore, IL-21 also inhibited the Cp RNAs already at 12 h in the type III BL lines. Because the decay of both Cp and LMP-2A mRNAs started already at 9–12 h, when EBNA-2 protein was unchanged, IL-21 treatment may inhibit the transactivation function of EBNA-2. This would explain why both EBNA-2-regulated EBV promoters (Cp and LMP-2A) are inhibited in parallel.

STAT3 was shown to activate the TR LMP-1 promoter in both reporter assays (15) and epithelial cell lines (16, 17). Therefore, the induction of LMP-1 expression by IL-21 is probably mediated by STAT3. The relative contribution of the TR versus the ED-L1 in the aforementioned studies could not be addressed because the expression of TR– or TR+EDL1-derived LMP-1 expression was measured with an endpoint RT-PCR assay. Here we quantified the IL-21-induced LMP-1 RNAs with Q-PCR assays using two PCR primer pairs (with similar amplification efficiencies) that amplify either the

total LMP-1 mRNAs (TR+EDL1) or only the LMP-1 messages originating upstream of ED-L1, and found that the majority of the LMP-1 RNAs still originated from of the ED-L1 promoter.

We showed previously that IL-10 could induce the expression of LMP-1 in the absence of EBNA-2 in type I BL and NK lymphoma cell lines (23). Because both IL-10 and IL-21 potentially activated STAT3, the LMP-1-inducing effect of the two cytokines probably converges on STAT3. A further interesting parallel between the effect of IL-10 and IL-21 is the induction of LMP-1 in the absence of EBNA-2 in the estrogen-prestarved ER/EB2-5: not only that LMP-1 could be induced, but also that the cells transiently re-entered the cell cycle. This proliferation-inducing effect of the cytokines might be due to the combined effect of the cytokine-activated signals and the cytokine-induced LMP-1 mimicking the physiological T cell-dependent cytokine plus CD40L signals. The obvious difference between the two cytokines was noted with regard to their effect on the type III cells. Only IL-21 could induce the expression of Blimp-1 and repress the expression of the Cp-derived EBNA transcripts, and as such IL-21 treatment of the type III cells was the only in vitro system in which Cp activity could be repressed and Qp induced, whereas LMP-1 expression and cell proliferation could be maintained at will. Through the regulation of the major EBV latent promoters, IL-21 treatment induced a switch from a type III EBV gene expression to a type II-like expression. Although this switch seems to mimic the type III to type II switch proposed to occur during the differentiation of EBV-carrying naïve B to GC B cells (47), it is important to note that IL-21 induced Blimp-1 and PC differentiation, but not BCL6 and GC B cell differentiation. It remains to be tested whether in vivo the EBV-carrying type III B cells seen in the tonsils of healthy EBV carriers (47) or during primary infection (48) differentiate into Blimp-1-positive, type II-like plasmablasts.

IL-21 induced the maturation and enhanced cytotoxicity of NK and CD8⁺ T cells (49) and had antitumor effects in murine models (18, 49). In addition, IL-21 could induce apoptosis in chronic lymphocytic leukemia cells and other B cell lymphomas (49). Phase I and II clinical trials with IL-21 have been completed, providing data on the safety and efficacy in subjects with advanced melanoma, renal cell carcinoma, and non-Hodgkin B cell lymphoma (18). Based on our results, IL-21 could be considered for immunotherapy of EBV-carrying B cell malignancies. In type I B cell malignancies, IL-21 could induce the expression of LMP-1 that would provide new viral peptides recognizable by the cellular immunity. Furthermore, LMP-1 made the tumor cells more immunogenic (50, 51) and could directly inhibit the proliferation of BL cells (27). Taken together with its effect on the maturation and enhanced cytotoxicity of NK and CD8⁺ T cells, IL-21 has therapeutic potential in EBV-carrying lymphomas.

Materials and Methods

Cell Lines. The cell lines used were type I BL lines (Mutu I cl.59, cl.148, cl.216; Jijoye M13; Akata-Neo^R-converted DG75 BL sublines, DG75-Akata cl.2-2 and cl.6), type III BL lines (Mutu III cl.99, cl.176; Jijoye P79; AG876; BL16; BL18), and LCLs [the estrogen-dependent conditional LCL ER/EB2-5; the cord blood-derived LCLs CBM1-Ral-STO (with the Rael EBV strain) and CB-LCL-Jij.M13 (with the Jijoye M13 EBV strain); the tonsillar B cell-derived LCL TB-LCL-B958; the Nadia LCL]. More detailed information on the origin of the cell lines can be found in ref. 23. The ER/EB2-5 cells were kindly provided by G. W. Bornkamm (German Research Center for Environmental Health, Institute of Clinical Molecular Biology and Tumor Genetics, Munich). The EBV-negative lines Jurkat and MOLT-4 (T cell lymphoma lines), U266 (multiple myeloma line), and the EBV-positive type III BL line Raji were used as controls. All lines were cultured in RPMI medium supplemented with 10% heat-inactivated FCS, 1 mM L-glutamin, 100 units/mL penicillin, and 100 μ g/mL streptomycin, except for CB-LCL-Jij.M13, which was cultured in complete IMDM medium with 15% FCS. ER/EB2-5 cells were maintained in complete RPMI medium supplemented with 2 mM β -estradiol (Sigma-Aldrich).

Cytokines. The recombinant human IL-4, IL-6, IL-10, IL-15, IL-21, INF- α , and INF- γ were purchased from Peprotech. IL-2 was a gift of Ajinomoto Company.

IL-21 Treatment of Cells. The type I BL and the EBV-converted Ramos cells were cultured at 1×10^6 cells per 3 mL of complete RPMI medium and treated with 50 ng/mL IL-21 or the indicated doses. The LCLs and type III BL lines were cultured 0.5×10^6 cells in 3 mL of complete RPMI medium and treated with 100 ng/mL IL-21. For long-term treatments of LCLs the cells were replated every second day 0.5×10^6 cells per 3 mL with fresh IL-21 (100 ng/mL). For the IL-21 treatment the ER/EB2-5 cells were washed twice with PBS and plated in complete medium (1×10^6 cells per 3 mL) with 50 ng/mL IL-21. For the estrogen prestarvation experiments, cells were washed twice in PBS and

kept for 3 days without estrogen. Three days later the cells were counted and plated at 1×10^6 cells per 3 mL of complete RPMI with 50 ng/mL IL-21.

See *SI Experimental Procedures* and *Table S1* for more information.

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