B7-H1, Which Represses EBV-Immortalized B Cell Killing by Autologous T and NK Cells, Is Oppositely Regulated by c-Myc and EBV Latency III Program at Both mRNA and Secretory Lysosome Levels

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B7-H1, Which Represses EBV-Immortalized B Cell Killing by Autologous T and NK Cells, Is Oppositely Regulated by c-Myc and EBV Latency III Program at Both mRNA and Secretory Lysosome Levels

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EBV-immortalized B cells induce a complex immune response such that the virus persists as a clinically silent infection for the lifetime of the infected host. B7-H1, also called PD-L1, is a co-signaling molecule of the B7 family that can inhibit activated T cell effectors by interaction with its receptor PD-1. In this work, we have studied the dependence of B7-H1 on NF-κB and c-Myc, the two main transcription factors in EBV latency III proliferating B cells, on various lymphoblastoid and Burkitt lymphoma cell lines, some of them being inducible or not for the EBV latency III program and/or for c-Myc. We found that B7-H1 repressed killing of EBV-immortalized B cells by their autologous T and NK cells. At the mRNA level, NF-κB was a weak inducer whereas c-Myc was a strong repressor of B7-H1 expression, an effect mediated by STAT1 inhibition. At the protein level, B7-H1 molecules were stored in both degradative and unconventional secretory lysosomes. Surface membrane B7-H1 molecules were constitutively internalized and proteolyzed in lysosomes. The EBV latency III program increased the amounts of B7-H1-containing secretory lysosomes and their export to the surface membrane. By repressing actin polymerization, c-Myc blocked secretory lysosome migration and B7-H1 surface membrane export. In addition to B7-H1, various immunoregulatory molecules participating in the immunological synapse are stored in secretory lysosomes. By playing on actin polymerization, c-Myc could thus globally regulate the immunogenicity of transformed B cells, acting on export of secretory lysosomes to plasma membrane. *The Journal of Immunology, 2012, 189: 000–000.

Epstein-Barr virus is the first transforming virus described in humans. In vitro, this gamma-herpesvirus, belonging to the lymphocryptoviruses, infects and transforms primary B cells, leading to the continuous proliferation of lymphoblastoid cell lines (LCLs), corresponding to the so-called EBV proliferating program, or EBV latency III (expression of full range of EBV latent proteins). In vivo, EBV is also associated with various cancers, including nasopharyngeal carcinomas, Burkitt lymphomas, Hodgkin’s lymphomas, T cell lymphomas, and immunodeficiency-related B cell lymphomas, the latter directly caused by EBV (1).

Primary infections occur classically during infancy and are generally asymptomatic. However, some do have clinical symptoms, which manifest as the benign self-limiting lymphoproliferative disease infectious mononucleosis. During primary infection, EBV infects and induces continuous proliferation of resting B cells. In vivo, EBV-infected B cells are actively eliminated by a vigorous cytotoxic immune response, resulting in the spontaneous resolution of EBV primary infection. Yet, EBV persists silently in the memory B cell compartment of the organism throughout life and may be reactivated in case of immunodeficiency (2).

It has been shown that coevolution between EBV and its host has led to a selection of highly immunogenic EBV strains (3). Immunogenicity of EBV latency III infected B cells is due not only to expression of viral proteins presented by MHC molecules but also to cell proteins involved in the T cell synapse such as adhesion molecules (CD54, CD58, CD11a, or CD18), costimulatory molecules (CD80/B7-1 or CD86/B7-2), or cytokines favoring NK/T cell killing such as IL1, TNF-α, IL-2, IL-12, or IL-17 (4), as well as molecules whose activation induces apoptosis such as CD95 (5, 6). Despite vigorous immune responses leading to the killing of most EBV-immortalized B cells, the virus constantly reaches the memory B cell compartment where it is hidden from the immune system in a almost complete silent state. However, the virus remains continuously active in immunocompetent individuals even though at very low levels, leading to continuous virus excretion (7). Viral reactivations are due to stochastic induction of the lytic cycle during which both full-length and a truncated form of latent membrane protein 1 (LMP1), so-called lytic LMP1, are re-expressed (8, 9). Full-length LMP1 expression has been shown...
to play a critical role in virus production (10). Both the lytic cycle viral program and LMP1 expression are also known to be highly immunogenic. Immunogenicity of EBV-infected B cells is largely dependent on LMP1 expression and NF-κB activation, which in turn is responsible for IFN-γ secretion and STAT1 activation (11).

With NF-κB, c-Myc is the other master transcriptional factor of EBV latency III proliferating B cells (12). c-Myc overexpression may directly contribute to immune escape of tumor B cells through repression of the IFN response (13). Thus, from an immunological point of view, the two main transcription factors of EBV latency III proliferating B cells would act opposingly, as c-Myc would tend to decrease expression of active immunoregulatory surface molecules induced by continuous activation of NF-κB.

Among markers expressed by B cells that are involved in the T cell synapse and able, positively or negatively, to regulate T cell killing are molecules of the B7 family. The B7 family comprises seven molecules (14) including B7-H1 (CD274/programmed-death ligand 1; PD-L1). B7-H1 activation was initially ascribed to T cell proliferation, playing an important costimulatory role during primary T cell responses (15). Currently, the B7–H1–programmed-death 1 (PD-1) axis is thought to be a promising target for immunobiology of cancer therapies (16). B7–H1 may inhibit ongoing T cell responses by inducing apoptosis and arrest in cell cycle progression, by binding to PD-1 receptors on activated T cells (17). B7–H1 is a 290-aa type I transmembrane protein that contains extracellular IgV-like and IgC-like domains as well as a transmembrane domain (18). B7–H1 mRNA can be expressed in a variety of hematopoietic cell types, including APCs (B cells, macrophages, dendritic cells), T cells, monocytes, and NK cells, as well as in numerous nonhematopoietic cell types (epithelial, endothelial, pancreatic islet, mast, or mesenchymal) and tissues (heart, skeletal muscle, placenta, or lung) (14). Because of STAT1 activation and IFN regulatory factor 1 (IRF1) expression, IFNs are strong inducers of B7–H1 expression for either nonhematopoietic (19–21) or hematopoietic cell types (15, 22, 23). Aberrant high expression of B7–H1 has been reported in many cancers (melanomas, myelomas, lymphoproliferations, and breast, lung, ovary, and colon tumors) (16, 24), as well as during various chronic viral infections such as HIV, hepatitis C virus, or rhinovirus (16, 24). Similarly, B7–H1 expression is markedly increased in EBV-infected B cells (25).

In this study, we show that B7–H1 is involved in the killing of EBV-immortalized B cell by its autologous T and NK cells. We then describe the regulation of B7–H1 molecules in EBV-immortalized B cells and show that c-Myc and EBV latency III programs have opposing roles regarding B7–H1 regulation at both the mRNA and surface membrane levels.

Materials and Methods

Cell lines/cell models and chemical treatments

EBV-negative Burkitt lymphoma (BL) cell lines BL2 and BL41 and their EBV-positive counterparts, BL2B95.8 and BL41B95.8, as well as the lymphoblastoid cell line PRI were cultured in standard conditions as described (6, 26). The EBV-positive EREB2-5 cell line with an EBNA2-estrogen chimeric receptor was cultured in presence of 1 μM estradiol (Sigma-Aldrich, St. Louis, MO) (27). To arrest and reinduce the EBV latency III program, cells were deprived of estradiol for 72 h (resting cells, E−) and retreated with estradiol for 3 h, in the absence of or with 10 μg/ml isotypic control (purified mouse IgG1, κ) or B7–H1 blocking mAb (purified mouse mAb, clone MIH1; eBioscience). Cytotoxic effect on LCL B cells was evaluated by flow cytometry on CD19+ B cells (PE–CD19 mouse mAb, clone J3-113; Beckman Coulter) with the annexin V/TOPO-3 test, which identifies apoptotic cells (5).

RNA isolation and real-time quantitative PCR

RNA extraction and real-time quantitative PCR were performed as described previously (5). mRNA levels for B7–H1, TRAF1, c-Myc, TAP1, TRAF1, and IRF1 genes were quantified in parallel in an established gene expression system (Hs01125296_m1, Hs00194638_m1, Hs00153408_m1, Hs00388682_m1, Hs00233698_m1, and Hs99999901_s1, respectively; Applied Biosystems, Foster City, CA). Each PCR was performed in duplicate, which made it possible to assess cycle threshold (CT) values. For each sample, the mRNA relative expression level was calculated from C Ts as previously described (29). Briefly, mRNA levels for a given gene in a given sample were normalized to 18S RNA in the same sample as well as to mRNA levels of the given gene in a pool of control RNA from PBMCs of five healthy donors (relative mRNA expression level equal to 1 for the pool of control RNAs by definition).

Protein extracts and Western blotting

Total protein extracts were obtained as follows: 5 × 10^6 cells were resuspended in 100 μl Blue Læmmlí Lysis Buffer (Bio-Rad, Hercules, CA) and 5% 2-mercaptoethanol (Bio-Rad). Cyttoplasmic extracts were prepared as described (30). Ultracentrifugation at 100,000 × g for 1 h was performed to separate the cytosolic fraction (supernatant) from the organelles (pellet).

Western blotting was performed as described (31). Abs used were anti-B7–H1 (H310 rabbit polyclonal Ab; Santa Cruz) at 1/200, anti–c-α-tubulin (mouse mAb, clone B5.2.1; Sigma-Aldrich) at 1/10,000, anti-STAT1 (p84/p80) (rabbit polyclonal Ab; Santa Cruz) at 1/1000, anti-LAMP-1 (rat antibody Ab; Cell Signaling) at 1/1000, anti–LAMP-2 (mouse mAb, clone HA43; Santa Cruz) at 1/200, anti-LMP1 (S12 from hybridoma) at 1/100, and anti–c-Myc (mouse mAb, clone 9E10; Santa Cruz) at 1/200. Chemiluminescent revelation was performed using a standard protocol, as previously described (6).

Abs used for flow cytometry, confocal microscopy, and F-actin staining

The primary Abs used in this study were as follows: PE–CD274 (mouse mAb, clone 29E.2A3; BioLegend), PE mouse IgG2b, κ isotype control (BioLegend), goat polyclonal anti-cathepsin D (R-20; Santa Cruz), goat polyclonal anti–LAMP-2 (C-20; Santa Cruz), rabbit polyclonal anti-Tom 20 (FL-145; Santa Cruz), normal rabbit IgG and normal goat IgG (Santa Cruz), Rab27a (rabbit mAb, clone EPR3021; Euromedx), and CD63 (mouse mAb, clone MEM-259; Serotec). For indirect labeling, secondary Abs were conjugated with either Alexa Fluor 488, 594, or 633 (Invitrogen, Molecular Probes). For both flow cytometry and confocal microscopy, direct and indirect immunolabelings were performed on 50,000 cells as described (5). For flow cytometry intracellular staining, cells were fixed and permeabilized using the Intraprep kit according to the manufacturer’s protocol (Beckman Coulter). Intracellular staining for confocal microscopy was performed as follows: 500,000 cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed with PBS, permeabilized with 100 μl 0.1% Triton X-100 in room temperature, washed with PBS, and incubated with primary Abs (20 min at room temperature). Then, cells were washed with PBS and incubated with the corresponding secondary Abs for 20 min at room temperature. Finally,
cells were washed with PBS, F-actin was stained at room temperature with phalloidin conjugated with Alexa 488 (Invitrogen). Briefly, $5 \times 10^5$ cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, permeabilized with 0.5% Triton X-100 for 10 min, and washed with PBS. The pellet was then incubated with 1 µg phalloidin for 30 min, and finally cells were washed in PBS.

For flow cytometry, acquisitions were performed on a FACSCalibur flow cytometer (Becton Dickinson). Alexa Fluor 488 and Alexa Fluor 633 were excited with a 488-nm argon laser and a 633-nm helium–neon laser, respectively, and fluorescence was collected with 530 ± 15 nm and 661 ± 8 nm band-pass filters. Analyses were performed with CellQuest Pro software (Becton Dickinson).

For confocal microscopy, an additional nuclear counterstaining with Hoechst 33342 was performed. Images were obtained using a Zeiss LSM-510 laser scanning confocal microscope. Hoechst 33342, Alexa Fluor 488, and Alexa Fluor 594 were excited with a 405-nm laser diode, a 488-nm argon laser, and a 543-nm helium–neon laser, respectively. Fluorescence was collected with 420 ± 30 nm and 530 ± 15 nm band-pass and 650-nm long-pass filters.

Results

EBV latency III program increases B7-H1 expression at both transcriptional and cell surface levels

To compare B7-H1 expression between EBV-negative and EBV-positive B cells, we first studied B7-H1 relative mRNA expression levels in either EBV-negative or EBV latency III converted BL cell lines as well as in a classical LCL, PRI, and in the EREB2-5 cell line, a cell line with an estradiol inducible EBV latency III program (Fig. 1A). We observed that B7-H1 mRNA expression was much higher in EBV latency III positive than in EBV-negative B cells. Results obtained after estradiol treatment of EREB2-5 cells show that B7-H1 mRNA expression was induced by the EBV latency III program (Fig. 1A).

Because B7-H1 is known to be widely expressed on the cell surface of various types of immortalized cells, we compared plasma membrane expression of the EBV-negative BL2 and BL41 cell lines versus their EBV-positive counterparts (Fig. 1B). No surface labeling could be detected at the surface of EBV-negative cells. In contrast, B7-H1 expression was easily detected at the surface of EBV-immortalized B cells with a strong labeling. Thus, EBV immortalization led to an increase in both B7-H1 gene transcription and protein expression on plasma cell membranes of B cells.

B7-H1 is a negative regulator of anti-EBV autologous T cell cytotoxicity

To determine the role of B7-H1 in anti-EBV T cell responses, we established three different LCLs from three EBV seropositive volunteers. We then incubated CD2+ T and NK cells (SRBC AET cell purification) in the presence of LCL cells from the same donor (representative results are given in Fig. 2). Results showed that masking B7-H1 with a specific blocking Ab increased autologous T cell killing of EBV-immortalized B cells. This points out the inhibitory role of B7-H1 in EBV-related T cell-dependent cytotoxicity, as in other tumor cell models (16, 17).

NF-κB is weakly involved in the regulation of B7-H1 expression, and c-Myc is a negative regulator of mRNA and cell surface expression

Because NF-κB and c-Myc are the two major transcription factors of EBV latency III immortalized B cells (12), we asked whether B7-H1 expression would be dependent on these transcription factors. Chemical inhibition of NF-κB activation in EBV-infected cells resulted in a weak decrease in B7-H1 expression at the mRNA level only (Supplemental Fig. 1), with no significant changes in B7-H1 protein levels at the surface membrane (data not shown), suggesting that factors other than NF-κB participate in B7-H1 gene transcription regulation.

To investigate the role of c-Myc in the regulation of B7-H1 expression, we used the P493-6 B cell line. This cell line is de-
Effects of T and NK cells on CD19+ B cells (identified by immunofluorescence using a PE-conjugated mAb) were evaluated by flow cytometry using the annexin V/TOPRO-3 test. The number of early apoptotic cells (annexin V+ and TOPRO-3+ after the additional loss of their membrane integrity) was increased in presence of blocking anti-B7-H1 mAb, showing the protective effect of B7-H1 on LCLs. This result was representative of three independent experiments.

**FIGURE 2.** B7-H1 blocking of EBV-immortalized B cells increases T cell cytotoxicity. EBV-immortalized B cells were incubated with autologous T and NK cells from healthy EBV-positive donor, alone or with 10 μg/ml isotypic control or anti-B7-H1 mAb (clone MH1). Cytotoxic effects of T and NK cells on CD19+ B cells (identified by immunofluorescence with a PE-conjugated mAb) were evaluated by flow cytometry using the annexin V/TOPRO-3 test. The number of early apoptotic cells (annexin V+ due to the translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane), as well as late apoptotic cells (annexin V+ and TOPRO-3+ after the additional loss of their membrane integrity) was increased in presence of blocking anti-B7-H1 mAb, showing the protective effect of B7-H1 on LCLs. This result was representative of three independent experiments.

The B7-H1 gene regulatory region does not possess any binding site for c-Myc, whereas STAT1 and IRF1 (a target gene of STAT1) can directly bind to the B7-H1 gene promoter to increase its transcription (33). Because c-Myc is a negative regulator of STAT1 activation (13), we looked at regulation of known STAT1 target genes, IRF1 and TAP1, as well as at p-STAT1 (Tyr701) and STAT1 protein levels in P493-6 cells (Fig. 3C, 3D). As expected, c-Myc overexpression decreased mRNA expression of IRF1, TAP1, as well as activation of STAT1. In contrast, EBV latency III program led to STAT1 activation and increased expression of IRF1 and TAP1. Altogether, these results suggest that, due to STAT1 inhibition, c-Myc would repress B7-H1 expression at the mRNA level in a similar manner as IRF1 and TAP1.

**FIGURE 3.** c-Myc represses B7-H1 expression. (A) Relative mRNA expression levels of c-Myc and B7-H1 were analyzed by RT-PCR for the P493-6 line before (EBNA2 Off) and after induction (EBNA2 On) of the EBV latency III program by estradiol (1 μM for 48 h) and/or inhibition of c-Myc expression by tetracycline (100 ng/ml for 48 h). (B) Overlay of histograms for surface membrane labeling of B7-H1, as well as for the isotypic control. Each of the four cell conditions is indicated on the figure. The isotypic control corresponds to the black histogram on the left. (C) Relative mRNA expression of TAP1 and IRF1 (two STAT1 target genes) was assessed by RT-PCR for the same four conditions. (D) c-Myc, STAT1, and p-STAT1 (Tyr701) expression was analyzed by Western blot for the same four conditions. c-Myc represses B7-H1 at the mRNA level in a manner similar to that of repression of IRF1 and TAP1 through inhibition of STAT1 activation. For RT-PCR analysis, mean values ± SEM are the results of three independent experiments. Flow cytometric and Western blot analysis are representative of at least three independent experiments.
staining of the membrane (data not shown). In contrast, B7-H1 expression increased in the vesicular fraction of cells expressing the EBV latency III program. It is also of note for P493-6 cells in the EBNA2 Off state (Fig. 4B, lanes 1 and 2) that repression of c-Myc (EBNA2 Off/c-Myc Off resting condition) was associated with a marked increase in organelle-associated B7-H1 protein levels, suggesting that c-Myc could repress B7-H1 organelle localization in P493-6 cells.

**Intracellular B7-H1 is located in lysosomes**

We conducted a bioinformatic analysis of the B7-H1 amino acid sequence to identify consensus sequences indicative of its subcellular compartment localization (http://www.uniprot.org/uniprot/Q9NZQ7 and Fig. 4A). The N192 residue has been described as a genuine glycosylation site of B7-H1 (36). Further bioinformatic analysis revealed that B7-H1 possesses four asparagine residues, N35, N192, N200, and N219 (http://www.uniprot.org/uniprot/Q9NZQ7 and Fig. 4A). The N192 residue has been described as a genuine glycosylation site of B7-H1 (36).

**FIGURE 5.** Localization of intracellular B7-H1 in lysosomes. (A) B7-H1 contains a signal peptide (aa 1–18, red) including a tyrosine-based motif YWHL associated with sorting by conventional and/or secretory lysosomes. It also possesses extracellular Ig-V like (aa 19–127, dark blue) and Ig-C like (aa 133–225, green) domains, a transmembrane domain (aa 239–259, light blue), and a cytoplasmic tail (aa 260–290, pink). Underlined asparagine residues are described as glycosylation sites (N192 and N200, 219). (B and C) Colocalization of B7-H1 and lysosomal markers LAMP-2 and cathepsin D are shown for BL41 (B) and BL41B95.8 (C) cells. Anti-B7-H1 (mouse) and anti–LAMP-2 (goat) or anti-cathepsin D (rabbit) Abs were revealed with Alexa Fluor 488-conjugated anti-mouse or anti-rabbit (green) and Alexa Fluor 633-conjugated anti-goat or anti-rabbit Abs (red). Nuclei were counterstained with Hoechst 33342. A negative control was realized by costaining of B7-H1 and mitochondria with Ab against Tom 20 (revealed by Alexa Fluor 633-conjugated anti-rabbit Ab) (original magnification ×63). Isotypic controls were used to verify absence of nonspecific fluorescence (data not shown). Confocal analyses are representative of at least three independent analyses.
protecting lysosomal membrane proteins from degradation by the proteolytic environment in nondegradative secretory lysosomes (37), known to derive from the trans-Golgi network, representative of hematopoietic cells and used to convey membrane proteins to the cell surface (34).

The fact that most B7-H1 molecules seem to be located in the lysosomal compartment raised the question whether B7-H1 could be located in nondegradative secretory lysosomes. The amount of total lysosomes was assessed using the LAMP-2 marker whereas the secretory lysosomal compartment (34, 38) was estimated with the specific Rab27a marker, a small GTPase that participates in the docking of lysosome vesicles at the plasma membrane (34). A downward trend for EBV-immortalized B cells was observed for LAMP-2 compared with EBV-negative cells (Fig. 6A). In contrast, the lysosomal fraction associated with the Rab7a marker was significantly increased in EBV-immortalized B cells (Fig. 6B). The secretory lysosomal compartment appeared thus much more abundant in EBV-infected cell lines.

Therefore, we wanted to know if B7-H1 was located in secretory lysosomes. Colocalization experiments by confocal microscopy showed an overlap between B7-H1 and Rab27a (Fig. 6C). Part of the intracellular particles containing B7-H1 was not associated with Rab27a, which is consistent with the additional presence of the protein in conventional degradative lysosomes.

To assess B7-H1 lysosomal export to the membrane, we simultaneously quantified B7-H1 cell surface expression as well as both Rab27a and CD63 by flow cytometry (Fig. 6D). CD63 is a molecule that belongs to the tetraspanin family and plays a role in the regulation of cell development as well as cell activation and growth and is associated with the lysosomal compartment (39). Increased B7-H1 at the cell surface of EBV-infected cells paralleled Rab27a and CD63, reflecting enhanced fusion of secretory lysosomes with the plasma membrane. These results suggest that B7-H1 could be conveyed to the plasma membrane by secretory lysosomes, resulting in B7-H1 overexpression at the cell surface.

**FIGURE 6.** B7-H1 is associated with secretory lysosome content. (A) Total lysosomal content of BL2 and BL41 cell lines and their EBV-immortalized counterparts was estimated by intracellular labeling of LAMP-2 protein followed by flow cytometry. LAMP-2 mean fluorescence intensity ratios were calculated as the ratio between mean fluorescence intensity for LAMP-2 labeling and the isotypic control. (B) Total secretory lysosomal amount was evaluated as described earlier by intracellular labeling of the Rab27a secretory lysosomal protein. Mean values ± SEM are the results of three independent experiments. (C) Confocal microscopy of colocalization of B7-H1 (green) and Rab27a (red) for BL2 and BL2B95.8 cell lines. Nuclei were counterstained with Hoechst 33342 (original magnification ×63). (D) Plasma membrane expression levels for B7-H1 and for two markers resulting from the fusion of secretory lysosomes to the cell membrane, Rab27a and CD63, were estimated by flow cytometry. Mean values ± SEM are the results of three independent experiments. Confocal analysis and flow cytometric profiles are representative of at least three independent experiments.
c-Myc inhibits secretory lysosomal pathway and B7-H1 membrane expression by decreasing actin polymerization

Because we found that B7-H1 membrane expression was increased by c-Myc inhibition in the P493-6 model (Fig. 2B), we examined whether fusion of secretory lysosomes with the cell membrane was similarly affected. Flow cytometric analysis showed that Rab27a expression was significantly increased when the c-Myc program was switched off by tetracycline treatment (EBNA2 Off/c-Myc On and EBNA2 On/c-Myc On compared with EBNA2 Off/c-Myc Off and EBNA2 On/c-Myc Off, respectively) (Fig. 7A). Moreover, treatment of cells with 10058-F4 induced an increase in B7-H1 as well as Rab27a cell surface localization in all cell lines tested (Fig. 7B, 7C). As for B7-H1, Rab27a plasma membrane output was repressed by c-Myc. These results suggest that c-Myc is a negative regulator of secretory lysosome export to the cell membrane and therefore regulates B7-H1 surface expression.

Polymerized actin (F-actin) plays a crucial role in terminal events of vesicle externalization, a process closely regulated by small GTPase molecules of the Rho pathway (40, 41). c-Myc can block actin growth and polymerization by decreasing expression of numerous molecules of the actin network (actin capping protein, actinins, catenins, etc.) and the Rho family, such as RhoA, Cdc42, or Rock-1 (42–44). Fig. 8 shows that c-Myc expression decreased F-actin levels in P493-6 cells independently of the EBV latency III program (Fig. 8A). Similar results were observed for all cell lines tested after c-Myc inhibition (Fig. 8B). Latrunculin A was used directly to block actin polymeration. This treatment dramatically decreased both F-actin intracellular content (Supplemental Fig. 4A) and Rab27a surface membrane expression (Supplemental Fig. 4B). Thus, latrunculin A treatment inhibited membrane export of secretory lysosomes. It also led to a strong decrease in B7-H1 membrane expression even though cell protein content remained unchanged (Fig. 8C). These results indicate that secretory lysosome membrane export is indeed related to actin polymerization and that c-Myc represses membrane export of B7-H1 by blocking actin polymerization, which in turn inhibits vesicle membrane export.

Discussion

In this study, we show that B7-H1 is a regulator of EBV-immortalized B cell killing by T and NK cells and that regulation of B7-H1 expression in EBV-immortalized B cell killing takes place at various levels. At the mRNA level, NF-κB appeared to be a weak inducer whereas c-Myc was a strong repressor of B7-H1 expression, due to STAT1 inhibition. At the protein level, B7-H1 appeared to be stored in the lysosomal compartment, including both degradative and unconventional secretory lysosomes. Surface membrane B7-H1 was constitutively internalized in degradative lysosomes and underwent proteolysis. Finally, we found that the EBV latency III program induced export of B7-H1–containing secretory lysosomes to the surface membrane, a phenomenon that was repressed by c-Myc, an effect mediated by actin depolymerization.

Induction of B7-H1 expression has been reported for both cancers and chronic infection and is thought to participate in local immunosuppression through binding to its PD-1 receptor on T cells, giving an anergic signal (17, 18, 45). Transient immunosuppression has also been reported in patients with sepsis, and PD-1 null mice are markedly protected from the lethality of sepsis (46, 47). EBV primary infection corresponds to a transient state during which a vigorous cytotoxic answer is elaborated by the immune host against the virus, involving both T and NK cells. However, this infection is also associated with immunosuppression due partly to IL-10 secretion (48). Induction of B7-H1 at the surface membrane of EBV-infected cells is likely to play a role in the establishment of EBV infection in immunocompetent hosts (25). B7-H1 binding to PD-1 may induce IL-10 secretion (26), and indeed, we found that blocking B7-H1 increased the killing of EBV-immortalized B cells by cognate autologous T/NK cells.

Among cytokines reported to be responsible for an increase in B7-H1 expression is IFN-γ, which leads to STAT1 activation and then to IRF1 expression. In EBV latency III immortalized B cells, STAT1 constitutive activation is due to continuous NF-κB acti-
NF-κB activation in lymphoblastoid B cells may also lead to IFN-γ secretion and STAT1 activation. c-Myc has been described to counteract the effect of both NF-κB and IFN-γ (13) and to repress expression of immunoregulatory molecules (13). In agreement with Yamamoto et al. (52), we did not find strong evidence for NF-κB dependence on B7-H1 expression. In contrast, we found that c-Myc was a strong repressor of B7-H1 mRNA expression. This repression was concomitant with IRF1 and TAP1 mRNA decrease and was mediated by STAT1 inhibition. Moreover, c-Myc repressed B7-H1 protein export to the membrane. A role for c-Myc inhibition in the expression of immunoregulatory molecules has previously been reported (13) and is usually interpreted as a mechanism for immune escape of transformed cells. The fact that c-Myc also down-regulates B7-H1 slightly blurs this interpretation, as blocking of the B7-H1–PD-1 axis is supposed to enhance the T cell cytotoxic response (18, 45). However, B7-H1 activation can also enhance T cell proliferation and secretion of various cytokines such as IL-10, IFN-γ, and GM-CSF. B7-H1 also preferentially costimulates CD4+ T cells independently of CD28 and enhances mixed lymphocyte responses to allogenic Ags (15). Even if a negative regulatory role for B7-H1 has been documented in various human diseases, such as cancers or chronic viral infections, a positive regulatory role for B7-H1 has also been demonstrated in vitro and in various animal models, pointing out the complex interactions between B7-H1 and its receptors (45). The first steps of EBV-related B cell immortalization correspond to expression of EBNA2 and EBNA3P with only c-Myc induction and proliferation of infected B cells (48). It can be hypothesized that at the very beginning of EBV primary infection, c-Myc repression of B7-H1 expression could help the expansion of newly EBV-immortalized B cells by decreasing the initial T cell response against transformed cells. Expression of the full range of EBV latency III proteins 24 to 48 h later would then overcome c-Myc repression of B7-H1, and surface membrane B7-H1 molecules of EBV-immortalized B cells would help to limit the intensity of the chronic T cell response against the virus.

Analysis of the entire B7-H1 cell content revealed that B7-H1 protein was found at significant levels in both EBV-positive and EBV-negative B cells. This result repeatedly found by Western blot as well as by flow cytometry or confocal microscopy after intracellular labeling of B7-H1, and using various Abs, clearly suggested that B7-H1 is stored in the cytoplasm before being exported to the cell surface. A similar phenomenon has been described for immunoregulatory molecules such as MHC class II, CTLA-4, or BTLA (35, 53, 54). Other activating or proapoptotic molecules such as CD40L or FASL in T cells have also been reported to be stored in the cytoplasm (55, 56). One striking feature shared by all these immunoregulatory molecules is their association with the secretory lysosomal compartment, stressing the importance of these vesicles in the immune system. Through appropriate conditions of fixation, permeabilization, and immunolabeling, we showed for the first time, to our knowledge, that B7-H1 was also associated with the secretory lysosomal compartment in B cells. In that context, the EBV latency III program appears to be a positive regulator of the amount of secretory lysosomes as well as their export to the surface membrane. One explanation for the upregulation of this export could be the increase in Rab27a expression in EBV latency III infected B cells (57), as this small GTPase molecule is necessary for the final docking steps of granules at the immunological synapse (34). Another important step is the fusion between the lysosomal vesicle and the plasma membrane. This process is controlled by SNARE complexes in a calcium-dependent manner. LMP1, which is associated with constitutive in-
increased levels of intracellular calcium (58, 59), could indirectly contribute to the fusion of secretory lysosomes with the plasma membrane.

Genetic defects in secretory lysosome export such as Rab27a deficiency (Griscelli syndrome) or Chediak–Higashi disease (CHS1/LYST gene defect) are associated with neurologic problems, hypopigmentation, and immunodeficiency with EBV-associated lymphoproliferative disorders due to defects in neuro-mediator transport, accumulation of melanin in vesicles, and absence of peroxin export in NK and cytotoxic T cells, respectively, demonstrating the key role of these intracellular vesicles in cell biology (38, 60). c-Myc clearly has a negative effect on export of secretory lysosomal vesicles by blocking actin polymerization, explaining why non-B7-H1 expression was found at the cell surface of EBV-negative BL cells as well as in P493-6 cells in which only the c-Myc program was induced. This negative regulation of B7-H1 export is certainly an intrinsic property of c-Myc, as extinction of the c-Myc program in P493-6 cells or chemical repression of c-Myc in EBV-negative BL cells may also lead to increased B7-H1 expression of B7-H1. c-Myc is associated with both regulation of numerous genes involved in actin network (42–44) and various genes involved in regulation of cell immunogenicity (13). By blocking actin polymerization, c-Myc would decrease the export of secretory lysosomes and would thus act on one step of the regulation of various key molecules of the immunological synapse, providing a clue to the global regulation of the immunogenicity of c-Myc proliferating cells.

In summary, we demonstrated that B7-H1, which regulates T and/or NK cell killing of autologous EBV-immortalized B cells, is stored in secretory lysosomes and is continuously degraded in degradative lysosomes. The EBV latency III program and c-Myc play opposite roles on B7-H1 expression not only at the mRNA level but also at the membrane export level. This expression is mediated through upregulation or downregulation of both amounts and export at the plasma membrane of secretory lysosomes by EBV and c-Myc, respectively. Because in addition to B7-H1, various important immunoregulatory molecules involved in the formation of the immunological synapse are stored in secretory lysosomes, we hypothesize that controlling the amount and export of secretory lysosomes to cell membranes could be one of the means by which both c-Myc and EBV globally temporally regulate the immunogenicity of transformed B cells both at the very first steps of primary infection and when expansion of EBV-immortalized B cells takes place in vivo.

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Disclosures

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