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Type I interferons directly down-regulate BCL-6 in primary and transformed germinal center B cells: Differential regulation in B cell lines derived from endemic or sporadic Burkitt's lymphoma

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ABSTRACT

Type I interferons (IFN) exert multiple effects on both the innate and adaptive immune system in addition to their antiviral and antiproliferative activities. Little is known, however about the direct effects of type I IFNs on germinal center (GC) B cells, the central components of adaptive B cell responses. We used Burkitt's lymphoma (BL) lines, as a model system of normal human GC B cells, to examine the effect of type I IFNs on the expression of BCL-6, the major regulator of the GC reaction. We show that type I IFNs, but not IFNγ, IL-2 and TNFα rapidly down-regulate BCL-6 protein and mRNA expression, in cell lines derived from endemic, but not from sporadic BL. IFNα-induced down-regulation is specific for BCL-6, independent of Epstein–Barr virus and is not accompanied by IRF-4 up-regulation. IFNα-induced BCL-6 mRNA down-regulation does not require *de novo* protein synthesis and is specifically inhibited by piceatannol. The proteasome inhibitor MG132 non-specifically prevents, while inhibitors of alternate type I IFN signaling pathways do not inhibit IFNα-induced BCL-6 protein downregulation. We validate our results with showing that IFNα rapidly down-regulates BCL-6 mRNA in purified mouse normal GC B cells. Our results identify type I IFNs as the first group of cytokines that can down-regulate BCL-6 expression directly in GC B cells.

1. Introduction

Affinity maturation is defined as the gradual increase in the affinity of serum antibodies following infection or immunization. This process occurs in the germinal center (GC) as the result of random somatic hypermutation of B cell receptor (BCR) genes followed by selection of B cell clones with increased affinity for antigen [1]. Despite the importance of affinity-based selection in normal antibody-mediated immunity, the mechanisms that regulate this process within the GC are not completely understood.

BCL-6 is a master regulator of the GC reaction. It is a transcriptional repressor belonging to the BTB-POZ zinc finger family, expressed only in GC B cells and small resting pre-BII cells in the B cell lineage [2,3]. The importance of BCL-6 in the formation of GCs and the development of normal T cell-dependent humoral immune responses was shown in BCL-6-null mice, which do not form GCs and are therefore unable to produce high-affinity antibodies [4,5]. In GC B cells BCL-6 modulates activation and apoptosis, in addition to controlling DNA-damage sensing and response [2]. Furthermore, downregulation of BCL-6 expression at the late stages of the GC reaction is critical for the exit of B cells from the GC and their differentiation into memory and plasma cells [6,7]. BCL-6 exerts these effects via the repression of more than 1200 target genes, with a broad control of several targets along the same pathway [2,8]. Several signaling pathways, known for their involvement in the GC reaction, have been shown to modulate BCL-6 expression both at the transcriptional and protein level. Activation of BCR by the antigen induces mitogen-activated protein kinase (MAPK)-mediated, while DNA-damage accumulation leads to ATM-promoted phosphorylation of BCL-6 protein, and thereby inducing subsequent BCL-6 degradation by the

Abbreviations: AID, activation-induced cytidine deaminase; BCR, B cell receptor; BL, Burkitt's lymphoma; CD40L, CD40 ligand; CHX, cycloheximide; eBL, endemic BL; EBV, Epstein–Barr virus; ERK, extracellular signal regulated kinase; GC, germinal center; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IRF, interferon regulatory factor; ISG56, IFN-stimulated gene 56; ISGF3, IFN-stimulated gene factor 3; MAPK, mitogen-activated protein kinase; OBF-1, OCT-binding factor 1; PCR, polymerase chain reaction; PIC, piceatannol; PKC, protein kinase C; RT-PCR, reverse transcription PCR; sBL, sporadic BL; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor.

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ubiquitin–proteasome pathway [9,10]. Stimulation of the CD40 receptor by CD40 ligand (CD40L) expressed on T cells leads to NF-κB-mediated transcriptional activation of interferon regulatory factor 4 (IRF-4), that directly represses BCL-6 transcription [11]. In addition to transcriptional down-regulation, exposure of GC B cells to CD40L rapidly disrupts BCL-6-corepressor complexes, leading to the rapid induction of BCL-6 target genes [12].

Burkitt's lymphoma (BL) is a high-grade B cell lymphoma, with an aggressive clinical course and a high proliferative rate. BL is characterized by chromosome translocations between the proto-oncogene c-myc and one of the immunoglobulin (Ig) loci. BL occurs sporadically (sBL) in the West, but was originally recognized in its endemic form (eBL) in equatorial Africa. Several differences have been found between these two subgroups, including age distribution, primary localization, sensitivity to chemotherapy, location of the myc and Ig breakpoints in the driving Ig/myc translocation and Epstein–Barr virus (EBV) status, as eBL is almost always associated with EBV, whereas sBL has a more irregular association, ranging from 10% to 30% positivity in different areas [13]. Additionally, molecular analysis of rearranged V_H genes showed a low number of somatic mutations and no signs of antigen selection in sBLs, in contrast to eBLs that showed more somatic mutations and signs of antigen selection [14]. It has been suggested that sBLs originate from early centroblasts that have gone through only one round of somatic hypermutation, while eBLs may originate either from late GC B cells or post-GC memory B cells [13,14]. A recent study showed however, that molecular profiles of all BL types were significantly more related to those of GC lymphocytes than to those of memory and naive B cells, and gene set enrichment analysis also did not show any evidence of possible enrichment in either memory or plasma cell programs in eBL cells [15]. Furthermore, c-Myc over-expression in an *in vitro* model of BL (although precipitated expression of GC surface markers) induced only substantially lower BCL-6, E2A and activation-induced cytidine deaminase (AID) protein levels, compared to BL cells and somatic hypermutation was not induced [16]. These observations, therefore argue for a GC origin of eBL, rather than c-Myc induced phenotypic change of non-GC B cells, and provide evidence that both major subgroups of BLs can be used as a model system of normal human GC B cells.

Type I interferons (IFN) are produced in relatively high amounts in response to pathogen sensing by the innate immune system [17]. In addition to their direct antiviral activities, these proteins also have antiproliferative effects by inhibiting cell growth or inducing apoptosis [18]. Furthermore, type I IFNs exert multiple direct and indirect effects on both the innate and the adaptive immune system [17]. Little is known, however about the direct effects of type I IFNs on GC B cells, the central components of adaptive B cell responses. Since normal GC B cells cannot be cultured for longer periods *in vitro* due to rapid induction of apoptosis in the absence of CD40 stimulation [10,11], we used BL lines as a model system, to study in GC-derived B cells the effect of type I IFNs on the expression of BCL-6, the major regulator of the GC reaction whereas purified mouse normal GC B cells were utilized only for the validation of the results.

2. Materials and methods

2.1. Human cell lines and cell culture

The following human cell lines were studied: BL2, BL28, BL31, BL32, BL41, Ramos and DG75 are EBV-negative BL lines derived from sBL; P3HR1, Daudi, Rael, Akata and Mutu-BL-I-cl-148 are EBV-positive lines derived from eBL, while Mutu-cl-9, Mutu-cl-30 and Akata-cl-26-43 are originally EBV-positive eBL-derived lines that have lost the virus. The characteristics of these cell lines are listed in Table 1. CBM1-Ral-STO is a cord blood-derived lymphoblastoid cell line, transformed with the Rael EBV strain [24]. Farage is an EBV positive, diffuse large B cell lymphoma [29] and Jurkat is a human T cell acute lymphoblastic leukemia cell line [30]. All human cell lines were cultured in RPMI medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin.

2.2. Purification of mouse GC B cells

C57BL/6 female mice were purchased from Charles River and were allowed to acclimatize for at least 1 week. They were 2–6 months old when used in experiments. Mice were immunized 1–4 times with sheep erythrocytes, diluted in balanced salt solution. Spleens were removed on day 6 after the last immunization. GC B cells were purified using negative selection (based on the protocol of Cato et al. [31], with minor modifications) to remove cells positive for CD3, CD11c, CD43 and IgD (eBioscience and BD Biosciences) with the help of a biotin selection kit from StemCell Technologies. Degree of enrichment was assayed in a FACS Calibur after staining cells with anti-B220-APC and anti-GL7-FITC (both antibodies from BD).

2.3. Treatment with cytokines and inhibitors

Human IFNα, IFNβ, IFNγ, TNFα, IL-6 (all from PeproTech), IL-2 (gift of Ajinomoto Company) and mouse IFNαA (PBL InterferonSource) were diluted as recommended by the manufacturer, and frozen in aliquots. Human cell lines were plated at 1×10^6 (or 3×10^5 for the 3 days IFNα treatment) cells/ml/well of RPMI–10% FCS medium and treated for the times and with the concentrations of recombinant human cytokines as indicated. Purified mouse GC B cells were plated at 1×10^6 cells/ml/well in complete RPMI medium supplemented with sodium-pyruvate, 2-mercaptoethanol and 10% FCS and left untreated or treated with 500 U/ml IFNαA for 4 h.

Cycloheximide (CHX; 100 mg/ml stock; Sigma–Aldrich), Z-Leu-Leu-Leu-CHO (MG132; 25 mg/ml stock; Enzo), piceatannol (PIC; 10 mg/ml stock; Sigma–Aldrich), Wortmannin (10 mM stock; Calbiochem), 2′-amino-3′-methoxyflavone (PD98059; 5 mg/mL stock; Calbiochem), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB203580; 10 mg/ml stock; Calbiochem), bisindolylmaleimide I (Gö6850; 5 mM stock; Calbiochem), (E)3-[(4-Methylphenyl)sulfonyl]-2-propenenitrile (BAY117082; 100 mM stock; Calbiochem) and 6-amino-4-(4-phenoxyphenylethylamino)-quinazoline (QNZ; 10 mM stock; Calbiochem) were dissolved in DMSO, while the NF-κB SN50 cell-permeable inhibitory peptide (SN50; 2.5 mg/ml stock; Enzo) was dissolved in water. For the treatments with the inhibitors, 1×10^6 cells/ml/well in 48-well (Daudi) or 24-well (Mutu-cl-30) plates were preincubated with the indicated concentrations of inhibitors or the same volume of DMSO for 30 (CHX, PD98059 and Gö6850), or 45 (MG132, PIC, SB203580, BAY117082, QNZ and SN50), or 60 (Wortmannin) min, after which 20 ng/ml IFNα was added (or the cells were left untreated), and incubated for an additional 3 or 6 h as indicated, when RNAs and total cell lysates were prepared.

2.4. Immunoblotting

Total cell lysates were prepared and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting, with the following antibodies: mouse anti-β-actin (AC-15; Sigma–Aldrich), mouse anti-BCL-6 (D-8; Santa Cruz Biotechnology), mouse anti-IRF-4 (MUM1p; Dako), goat anti-Pax-5 (C-20; Santa Cruz Biotechnology), rabbit anti-STAT-5 (N-20; Santa Cruz Biotechnology) and rabbit anti-phospho-STAT-5 (Tyr694; Cell