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Stat3 Is Important For The Emergence Of Human B Cells Predicted To Proliferate After Infection With Epstein-Barr Virus

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STAT3 IS IMPORTANT FOR THE EMERGENCE OF HUMAN B CELLS PREDICTED TO PROLIFERATE AFTER INFECTION WITH EPSTEIN-BARR VIRUS

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Many unanswered questions surround the early changes in B cells that lead to establishment of latency and immortalization following Epstein-Barr virus (EBV) infection. Data from the Bhaduri-McIntosh lab revealed that EBV differentially infects B cells into distinct sub-populations, only one of which, those marked $CD23^{hi}$ $CD58^{+}$ $IL6^{-}$, can be predicted to proliferate as early as 3 days after exposure to EBV. Other cells produced IL6 but did not proliferate, perhaps assisting a select few to do so. They also found that high levels of signal transducer and activator of transcription 3 (STAT3) correlate with resistance to lytic cycle induction and hence maintenance of the immortalized state. We hypothesized that 1) IL6 is necessary for the emergence of $CD23^{hi}$ $CD58^{+}$ B cells following infection with EBV, and that 2) phosphorylation of STAT3 is necessary for the emergence of this B cell sub-population that is predicted to proliferate. We tested these hypotheses and showed that blocking IL6 by infecting human primary B cells with EBV in the presence of neutralizing antibodies did not have an effect on the emergence of $CD23^{hi}$ $CD58^{+}$ cells. However, the STAT3 inhibitors Stattic and AG490, abrogated the emergence of this sub-population, especially within 24 hours of exposure to EBV. We concluded that phosphorylation of STAT3 is important for the emergence of human B cells predicted to proliferate during the process of immortalization after EBV infection.

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And last, but certainly not least, I thank my family for being with me throughout this journey, as they have done so time and time again. Home-cooked Filipino meals, laughter, and yes, plenty of distraction, can reinvigorate the spirit like no other.

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INTRODUCTION

The Epstein-Barr virus (EBV) was discovered in 1964 by Epstein, Achong, and Barr from electron micrographs of cells cultured from Burkitt's lymphoma (BL) tissue after Denis Burkitt, a British colonial surgeon in Uganda, had raised the possibility of an infectious etiology for the multifocal jaw tumors he encountered in children throughout sub-Saharan Africa following observations of the lymphoma's overlapping distribution with endemic malaria (1). Four years later, serendipitously, E.H., a research technician working in a virology laboratory, became EBV seropositive after contracting infectious mononucleosis (IM), providing the main clue that led to further investigations identifying the virus as the cause of heterophile-positive IM (2). Subsequent studies demonstrated the lymphoproliferative effect of EBV, transforming B cells *in vitro* into continuously proliferating lymphoblastoid cell lines (LCLs), a state that has since been termed "immortalization" (3, 4), further establishing EBV as the first human virus implicated in oncogenesis (5).

Since its original description 46 years ago, EBV has been associated with a heterogeneous group of malignant diseases (6, 7). Its genome has been detected in tissue biopsies from patients with nasopharyngeal cell carcinoma (NPC) (8); within the epithelial cells of oral "hairy" leukoplakia in AIDS patients (9); in T cell lymphomas (10); and in Reed-Sternberg cells of Hodgin's disease (11). The virus has also been firmly linked as the pathogenic agent in a variety of other human tumors, including non-Hodgkins lymphomas and lymphoproliferative disease (LPD) in immunodeficient hosts (e.g. AIDS-associated, post-transplant, and nasal T/natural killer cell), while its

association with leiomyosarcomas in immunosuppression is suspected (6, 12). Although the majority of people infected with EBV will not develop cancer as a consequence of their infection (13), the elevated incidence of some of these diseases in certain populations (6) and the rise of organ transplantations and immunosuppressive therapy for various conditions necessitate a better understanding of the oncogenic potential of EBV and its role in the pathogenesis of this heterogenous group of cancers.

EBV and its natural history of infection

EBV is a gamma-herpes virus that infects >90% of the world's population, benignly persisting for the host's lifetime (1, 6, 14, 15). This persistent infection is evident as stable levels of viral shedding in saliva (16). Infectious virus can be detected in oropharyngeal secretions from nearly all seropositive individuals (16). Persistently infected individuals also demonstrate infected B cells in the blood (17) and lymphoid tissue (12). Infection usually occurs via contact with oral secretions; however, blood transfusion and bone marrow and organ transplantation have also been implicated (18). In developing countries and lower socioeconomic groups, presumably due to socio-demographic factors controlling exposure to the virus (19), primary infection with EBV usually occurs during the first few years of life and is often asymptomatic or manifests as a non-specific illness. On the other hand, primary infection is more frequently delayed until adolescence or young adulthood in industrialized countries and higher socioeconomic groups, producing the characteristic clinical features of acute IM in 50% of such individuals (2, 15, 20).

The EBV genome is composed of linear double-stranded DNA encoding approximately 100 viral proteins (6, 7, 15). Infection with EBV, like that with other herpes viruses, involves both lytic and latent phases (6). Two target cell types can be infected: oropharyngeal epithelium and resting or non-proliferating, memory B lymphocytes (21, 22). Infection of epithelial cells by EBV results in active replication leading to release of infectious virions into saliva (15). This replicative phase, also known as productive or lytic infection, involves expression of the full array of nearly 100 viral lytic cycle genes (9, 23). In contrast, EBV persists in latently infected, resting, memory B lymphocytes in the blood (24). *In vitro* infection of B cells leads to their immortalization (3, 22). Upon infection of B cells, the linear EBV genome becomes circular, forming an episome, and usually remains latent, with viral replication spontaneously activated in only a small percentage of cells (15). In healthy, persistently infected individuals, approximately 1 in 10,000 to 100,000 memory B cells in the circulation are infected with EBV, and the number of latently infected cells remains stable over years (12, 22).

The establishment of latency with periodic episodes of viral lytic replication within the long-lived memory B cell compartment appears central to the phenomenon of viral persistence, resulting in a controlled lifelong EBV infection despite sustained virus-specific cellular and humoral immune mechanisms (12). However, with immunosuppression, a decline in effective T-cell surveillance can lead to virus reactivation, elevated blood and plasma levels of EBV-DNA (14, 25, 26), and increased

numbers of latently infected B cells in the peripheral blood (14, 27). These events can then promote uncontrolled EBV-driven lymphoproliferation and development of a tumor (12).

Persistence of EBV in B cells

Latent infection within the B cell compartment in healthy EBV-seropositive individuals is characterized by the limited expression of a subset of virus latent genes (7). A minimal form of latency, type 0, is characterized by the expression of a single viral gene product, LMP2A. Lack of expression of nearly all EBV genes allows B cells to evade the host cell-mediated immune response (21, 28). Resting, memory B cells in type 0 latency persist for life in the peripheral blood of healthy individuals. Latency types 1 and 2 are characteristically observed among tumor cells in specific EBV-related diseases such as Burkitt lymphoma (type 1 latency), Hodgkin's disease (type 2 latency), and nasopharyngeal cell carcinoma (type 2 latency). Latency type 3 is characterized by the expression of all nine viral latency proteins. These include the nuclear antigens (EBNA 1, 2, 3A, 3B, 3C, and Leader protein) and the latent membrane proteins (LMP 1, 2A, and 2B). In addition, two sets of non-translated small RNAs (EBERs 1 and 2, and BART) are also expressed from the EBV genome (21). Latency type 3 is characteristic of LCLs, i.e. EBV-immortalized B cells *in vitro*. LCL-like cells are strongly immunogenic *in vivo*, and therefore readily recognized and controlled by cytotoxic T lymphocytes. The resulting expansion of cytotoxic T cells directed against cells in which viral replication has been activated is the main component of the classic lymphocytosis of IM (12). B cells similar

to LCL with a type 3 latency pattern are characteristic of tumor cells in LPD and are also found in tonsils of healthy EBV-seropositive individuals (29). While LCL generated *in vitro* from healthy human subjects have been used to study EBV gene expression, its persistence in B cells, and the role of the virus in tumorigenesis, little is known about cellular processes that occur during early stages of infection with EBV.

EBV-driven B cell proliferation

As mentioned previously, EBV can transform or immortalize human B lymphocytes *in vitro* to a state of continuous proliferation, generating permanent LCLs containing multiple copies of the viral genome in the form of episomes in every cell (30). Given that only a few genes are characteristically expressed in the latently infected, growth-transformed lymphocyte despite the presence of the complete EBV genome, these genes were investigated to examine their likely role in viral persistence and EBV lymphoproliferation (31). Initial studies of B cell immortalization by EBV were performed by infecting peripheral B cells, converting EBV-negative BL cell lines into EBV^+ cell lines by infection, or transfecting EBV-negative BL cell lines with EBV genes (4, 32, 33, 34, 35). These early experiments led to the characterization of two primary transforming proteins encoded by EBV: Latent Membrane Protein (LMP) 1 and Epstein-Barr Nuclear Antigen (EBNA) 2 (36).

LMP1 was found able to transform rodent fibroblasts and increase tumorigenicity in nude mice (34), providing evidence of its key role in EBV-induced proliferative transformation. When introduced by gene transfer into EBV-negative BL cell lines,

LMP1 induced broad phenotypic changes, altering cell morphology and increasing proliferative ability. These LMP-1 expressing cells also exhibited increased homotypic adhesion as a result of increased expression of the adhesion molecules LFA1 (CD18) and ICAM1 (CD54), thought to foster B cell growth by enhancing autocrine growth factor effects. In addition, LMP1 induced CD58 (LFA3) expression, which, alongside ICAM1, results in increased susceptibility of EBV-infected cells to immune surveillance via heterotypic adhesion to T lymphocytes. Such an effect is believed to permit EBV to establish a commensal relationship with the host as a repository for infectious virus (31).

Several lines of evidence also implicate EBNA2 as at least one other EBV protein necessary for EBV lymphoproliferation. Infection of EBV-negative BL cell lines with P3JHR1, an EBV strain with a deletion within a region of the genome containing the coding sequence for EBNA2, did not result in immortalization (32). EBNA2 also specifically induces expression of CD23, a marker of immortalization, following EBV infection and when transfected into B cells (35, 36).

IL6 may be important in assisting cells predicted to proliferate

Early experiments of EBV-mediated B cell immortalization singled out CD23, the low affinity receptor for IgE, as an early marker of EBV infection (4). Compared to activation of B cells by other mechanisms, EBV infection induced higher levels of CD23 (33). While expression of CD23 on EBV-infected cells expressing Epstein-Barr nuclear antigens was found to be necessary for immortalization (4), it is unclear if expression of both CD23 and latency antigens is sufficient for immortalization. Indeed, early

experiments by Thorley-Lawson and Mann showed that there were more cells that were infected ($EBNA^+$) than those that also expressed CD23 and progressed to become a line of established proliferating lymphoblasts (4). This observation is consistent with findings from recent studies done in the Bhaduri-McIntosh lab demonstrating that only one sub-population of EBV-infected B cells proliferates from at least two that newly emerge after infection with EBV (Megyola *et al*., submitted manuscript).

By correlating expression of a battery of molecular markers of host cell origin that have been described following encounter with EBV or viral proteins, such as CD18, CD58, CD54 (100), IL6, IL10 (10, 96), HLA class II, CD21, CD80, CD86, PD1, and CD57 with expression of CD23, expression of EBV latency genes, and B cell proliferation, Dr. Bhaduri-McIntosh discovered that EBV differentially infects primary B cells into sub-populations of cells. Only a single sub-population of infected cells proliferates. Proliferation is a prerequisite for immortalization to occur. Cells predicted to proliferate can now be identified as early as 3 days following exposure of primary B cells to EBV.

Through *ex vivo* experiments using primary B cells from four healthy EBV-seropositive adults, they showed that CD58, in combination with CD23, is an early marker for EBV-mediated proliferation of B cells, and that following exposure of B cells to EBV, four sub-populations of cells are observed, differentiated by expression of CD23, CD58, and IL6: a $CD23^{hi}$ $CD58^+IL6^-$ group of cells that continues to proliferate indefinitely, a $CD23^{lo}$ $CD58^+$ group that expresses IL6 but does not proliferate, a $CD23^{lo}$ $CD58^-$ group and a $CD23^-$ group that neither express IL6 nor proliferate. Most notably,

while $CD23^{hi}$ $CD58^{+}$ and $CD23^{lo}$ $CD58^{+}$ cells both express EBV latency genes including LMP1 and EBNA2, only $CD23^{hi}$ $CD58^{+}$ cells undergo proliferation. No differences in the levels of expression of LMP1 protein or the fraction of cells expressing LMP1 protein were observed between the two sub-populations. Since sorted $CD23^{hi}$ $CD58^{+}$ $IL6^{-}$ cells did not proliferate in culture in the absence of the other sub-populations, it is plausible that the non-proliferating sub-populations of cells share a common goal: to potentiate proliferation of a select few marked as $CD23^{hi}$ $CD58^{+}$ $IL6^{-}$. Indeed IL6 present in mixed infected cultures may play a role in assisting cells destined to proliferate. In this study, we sought to examine this potential role further.

1L6, STAT3, and human tumors

IL6 is a cytokine produced by monocytes, B cells, fibroblasts, endothelial cells, and other cell types. Its multiple functions include its important role in the proliferation and maturation of B cells (37). Overproduction of IL6 is thought to be involved in the pathogenesis of lymphoid malignancies and high-grade B-cell lymphomas, with the cytokine acting as a growth factor for malignant B cells including multiple myeloma (38) and EBV-transformed B cells (39, 40, 41, 42). In addition, IL6 can induce latent EBV to undergo lytic replication in B cells (43) while EBV can induce B cells to produce IL6 and its receptor (44), further facilitating tumorigenesis.

Evidence suggestive of the role of IL6 in EBV lymphomagenesis is seen in both animal models and humans. Severe combined immunodeficient (SCID) mice transplanted with EBV^{+} human peripheral blood leukocytes (hu-PBL) developed LPD of

human B cell origin, manifesting as large lymphoid tumors characteristic of aggressive LPD seen in immunosuppressed patients (45, 46, 47). Of note, these xenotransplanted SCID mice also had detectable serum levels of human IL6 (48, 49). Subsequent experiments using the same model demonstrated a significant decrease in tumor incidence and improved survival in hu-PBL-SCID mice after inhibition of human IL6 with a neutralizing monoclonal antibody (50). In humans, abnormally high levels of IL6 are observed in patients with LPD (37, 40). Haddad *et al*. then showed that monoclonal anti-IL6 antibody treatment of 10 patients with transplant-associated LPD refractory to reduction of immunosuppression resulted in complete remission in 5 patients and partial remission in 3 (37). In the face of increasing evidence implicating the cytokine as an important factor in a variety of hematological and epithelial malignancies, it is no surprise that IL6 can also induce one of the signal transducers and activators of transcription (STATs), specifically STAT3, a protein that is itself associated with oncogenesis.

STATs comprise a family of structurally related cytoplasmic proteins originally identified as key intermediaries in cytokine-dependent signaling pathways and recognized for their dual function: signal transduction in the cytoplasm and activation of transcription in the nucleus, hence the name STAT (51). They participate in normal physiological cell processes such as cell survival, proliferation, differentiation, and apoptosis following activation by cytokines belonging to the IL6 cytokine family (52) and growth factors such as EGF and PDGF (53). STAT3 is one of seven STAT family members (STAT1, 2, 3, 4, 5A, 5B, and 6) that are activated via tyrosine phosphorylation

by members of the Janus kinase (JAK) family of protein tyrosine kinases in response to the aforementioned extracellular stimuli. The binding of ligand to the common receptor gp130 results in phosphorylation of STAT3 on tyrosine (Y) residue 705, leading to the formation of a dimer via the reciprocal interactions between the SH2 domain of one monomer and the phosphorylated tyrosine of the other (54). Dimerized STAT3 then translocates to the nucleus and directly binds to elements in the promoters of specific cellular genes essential in survival, proliferation, and development, regulating their transcription (53, 55). Aberrant STAT activity may thus contribute to malignant transformation by promoting cell proliferation and survival.

Constitutively activated STAT3 has been widely reported in many human cancer cell lines, leukemias and lymphomas, and solid tumors, including hepatocellular carcinomas, breast cancers, and head and neck cancers (56). In addition, studies have shown that constitutive activation of STAT3 occurs in cell lines transformed with v-Src, the transforming protein of Rous sarcoma virus; v-Abl, the oncogene transduced by Abelson murine leukemia virus; and by human T-cell leukemia virus-1 (53, 57, 58). Constitutive activation is not due to mutations in STAT3 itself but rather to deregulation of tyrosine kinases or of the ligands that activate STAT3 (53, 58, 59). In normal cell signaling, STAT activation is transient, under negative regulation produced by dephosphorylation of intermediates by protein tyrosine phosphatases, by the suppressor of cytokine signaling (SOCS) family of JAK inhibitors, and by the induction of STAT inhibitors such as the protein inhibitor of activated STAT (PIAS) (60). STAT3 activation that results in oncogenesis, however, is long-lasting and persistent, driving increased

expression of genes encoding the anti-apoptotic proteins Bcl-2, Bcl-xL, and mcl-1 (56), as well as cell cycle regulatory proteins that promote cell cycle progression such as c-Myc (61) and Cyclin D1 (54) and other transcription factors like c-Jun and c-Fos (62).

Phosphorylated STAT3 (P-STAT3) itself can drive the expression of *Stat3* gene. Indeed, a high level of expression of total STAT3 temporarily follows activation of STAT3 (56). *Stat3* is strongly upregulated in response to IL6, resulting in an increase in the amount of unphosphorylated STAT3 (U-STAT3) (55). Constitutive activation of STAT3 in many tumors leads to increased expression of U-STAT3, which in turn drives the expression of oncogenes such as MET and MRAS, which do not respond directly to P-STAT3 (55). A correlation between high levels of expression of *Stat3* and of these two oncogenes has also been demonstrated in some tumors using tumor tissue microarray (55).

Aberrant STAT3 activity also plays a role in EBV-driven tumorigenesis. Constitutively active STAT3 signaling is present in BL, NPC, Hodgkin's disease, and LPD (60, 63). EBNA1 (64) and LMP1 (60), the two latency proteins frequently expressed in EBV-associated tumors, have also been shown to be regulated by the JAK-STAT signal transduction pathway. LMP1 can also activate the expression of IL6 and in so doing promote phosphorylation at Y705 in an autostimulatory loop (65). Moreover, constitutively active STAT3 is required for the growth stimulatory and tumor-producing effect of LMP1 in an LMP1 transgenic mouse model (66).

Findings in the Bhaduri-McIntosh lab linked STAT3 levels to maintenance of latency in the BL-derived cell line HH514-16. Latently infected BL cells refractory to

EBV lytic cycle induction by sodium butyrate were confirmed to express high levels of STAT3 protein (67). Subsequent experiments found high levels of STAT3 expressed in LCL but not in uninfected, cultured primary B cells and showed that phosphorylation of STAT3 rapidly followed infection of primary B cells with EBV (Megyola *et al.*, unpublished data). While much is unknown regarding STAT3 and its role in the development and maintenance of EBV lymphomas, this study sought to investigate in particular its role, in conjunction with that of IL6, in the emergence of B cells predicted to proliferate during the immortalization process.

The association of IL6 and STAT3 with human cancers, in addition to the role of IL6 as a key intermediate in STAT3 activation, has raised interest in targeting them therapeutically. JAK family tyrosine kinase inhibitors and Src family kinase inhibitors block STAT3 activation and inhibit survival of human cancer cell lines (65). Peptides that block STAT3 dimerization and DNA binding activity have also been developed and shown to inhibit cell transformation (68). Targeting IL6 through antibody-mediated inactivation seems equally promising (37). It follows that a better understanding of the interactive roles 1L-6, STAT3, B cells, and EBV play in the promotion and maintenance of B cell proliferation following EBV infection may provide a rational basis for novel therapeutic interventions and lymphoma prevention, especially in vulnerable immunosuppressed populations.

HYPOTHESES AND SPECIFIC AIMS

Hypothesis 1: IL6 is necessary for the emergence of $CD23^{hi}$ $CD58^{+}$ B cells following infection of human primary B cells with EBV.

Specific Aim 1a: Use neutralizing antibodies to IL6 and/or IL6 receptor (IL6R) with isotype matched antibodies in parallel as control to determine if blocking the activity of IL6 will inhibit the emergence of the $CD23^{hi}$ $CD58^{+}$ B cell population after infection with EBV.

Hypothesis 2: Following exposure of human primary B cells to EBV, STAT3 is necessary for the emergence of $CD23^{hi}$ $CD58^{+}$ B cells that are predicted to proliferate.

Specific Aim 2a: Use STAT3 inhibitors (AG490 and Stattic) to determine if blocking the activity of STAT3 will abrogate the emergence of the $CD23^{hi}$ $CD58^{+}$ B cell population after infection with EBV.

Specific Aim 2b: Determine the window of time during which inhibition of STAT3 activity has an effect on emergence of $CD23^{hi}$ $CD58^{+}$ B cells.

MATERIALS AND METHODS

Statement of medical student contribution

The author prepared reagents, maintained cell cultures, and performed all experiments with the assistance of Cynthia Megyola and under the guidance of Dr. Bhaduri-McIntosh. EBV preparation and titration were performed by Cynthia Megyola. Phlebotomy and flow cytometry data acquisition and analysis were performed by Dr. Bhaduri-McIntosh.

Isolation of mononuclear cells and enrichment of B cells

3 healthy EBV-seropositive adults previously identified by Western blot analyses for antibodies to EBNA1 and small viral capsid antigen volunteered to donate blood for the study. Venous blood drawn in a heparin-coated syringe was diluted with 1 volume of RPMI 1640 medium (Sigma-Aldrich) and underlaid with 1 volume of lymphocyte separation medium (Ficoll-Hypaque; ICN). The mixture was centrifuged at 800xG for 40 minutes in a clinical centrifuge at room temperature. Peripheral blood mononuclear cells (PBMCs) were isolated from the interface of the gradient and resuspended at 2 x 10^6 cells/ml in complete RPMI 1640 medium containing 10% heat-inactivated, sterile filtered fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B. Since EBV persists in 1-50 out of 10^6 peripheral B cells (69), the likelihood of *in vitro* outgrowth of large numbers of proliferating endogenous infected B cells within a few days is minimal to none. T cells were removed prior to infection of B cells with EBV to minimize the effect of the cell-mediated immune system on EBV-driven B cell proliferation. To avoid non-specific expression of CD23 via inadvertent activation of B cells by bead-bound

antibodies used in positive selection procedures, a negative selection procedure was used to isolate B cells. PBMCs were subjected to $CD3^+$ cell depletion using immunomagnetic beads (Dynal; Invitrogen) using the manufacturer's instructions. Monocytes were not removed from the CD3-depleted cells since "accessory cells" would likely simulate an environment akin to *in vivo* infection for the B cells. In addition, more efficient outgrowth of EBV-infected B cells has been reported in the presence of auxiliary activation signals (70). However, when cells were harvested after culture with EBV, greater than 98% obtained for analysis were B cells because monocytes adhered to the plastic tissue culture wells, as has been described (Megyola *et al.*, unpublished data and 71). These experiments were performed with approval from the Human Investigation Committee at Yale University. Informed consent was obtained from the volunteers.

EBV preparation and titration

EBV-positive B95-8 cells were lytically induced with the inducing agent TPA for 1 hour followed by extensive washing and placement in culture. Cells were harvested after 4 days and EBV was isolated from the cell culture supernatant by centrifugation as described (72). Infectivity of the virus preparation was assessed by infecting BJAB cells, an EBV-negative B lymphoma cell line, with serial dilutions of virus. After 48 hours in culture, cells were examined for expression of EBNA by indirect immunofluorescence (73) and virus titer was calculated. For 2 different virus stocks, multiplicity of infection (moi) of 50-100 could be obtained with a 1:10 to 1:20 dilution of concentrated virus. The viral preparations were aliquoted and stored at -70°C.

IL6 and IL6R neutralization

CD3-depleted cells were incubated with rat monoclonal anti-IL6 neutralizing antibody (clone MQ2-13A5; BD Pharmingen) and/or mouse monoclonal anti-IL6R neutralizing antibody (clone B-R6; Abcam) at concentrations of 1 μg/ml, 10 μg/ml, and 100 μg/ml one hour prior to exposure to EBV and placed in culture at 37°C in the presence of 5% CO_2. A parallel set-up involving their respective isotype-matched antibodies, purified NA/LE rat IgG1κ (clone R3-34) and mouse IgG1 (clone NCG01), served as control.

STAT3 inhibition

One hour prior to exposure to EBV, CD3-depleted cells were incubated at 37°C in the presence of 5% CO_2 with either AG490 (Cayman Chemical) at 5-50 μM in DMSO as solvent, Stattic (Tocris Bioscience) at 10-50 μM, or DMSO at 5-50 μM as control. AG490 inhibits activation of STAT3 by inhibiting JAK2 (74) while Stattic is a non-peptidic inhibitor of STAT3 that inhibits binding of tyrosine-phosphorylated peptide motifs to STAT3 SH2 domain and inhibits STAT3 activation, dimerization and nuclear translocation (75). In subsequent experimental set-ups, CD3-depleted cells were exposed to EBV for 2 hours and placed in culture at 37°C in the presence of 5% CO_2, during which time AG490 at 25 μM was added 0-72 hours after EBV infection. After 2 hours of exposure to EBV, cells were washed 2 times with RPMI to prevent ongoing binding and infection with virus before they were replaced in culture. AG490 was replaced in the culture medium after washing.

Infection of B cells and cell culture

Following exposure of primary B cells with B95-8 virus derived EBV at moi of 50-100, cells were placed in culture at 37°C in the presence of 5% CO_2 at a concentration of 2 x 10^6 cells/ml in RPMI containing 10% FBS, penicillin, streptomycin, and amphotericin B. Cells were harvested on day 4 based on findings from the Bhaduri-McIntosh lab that markers for proliferating cells can be identified as early as 3 days following exposure of primary B cells to EBV. As previously mentioned, for the AG490 time point experiments the cells were washed twice with RPMI after exposure to EBV for 2 hours before they were replaced in culture.

Fluorescence-activated cell sorter (FACS) analysis

Cells were harvested at appropriate times and stained with saturating concentrations of fluorochrome-conjugated monoclonal antibodies against human cell surface molecules. Antibodies directed against surface molecules included anti-CD23-PE (BD Pharmingen) and anti-CD58-FITC (AbD Serotec). IgG from murine serum (Sigma-Aldrich) at 1 mg/ml was included in the mixture to inhibit non-specific binding. Cells were incubated with antibodies for 30 minutes at 4°C followed by 2 washes. Isotype matched control antibodies included monoclonal murine IgG1-PE and IgG1-FITC. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen). Cells were incubated for 30 minutes at 4°C with rat anti-IL6-APC (BD Pharmingen) or biotinylated anti-IL6 (eBioscience) and rabbit anti-STAT3 antibody (C20; Santa Cruz Biotechnologies) followed by 2 washes. Isotype control antibodies included monoclonal

rat IgG1-APC. Bound anti-IL6-Biotin antibody was detected using Avidin-PE-Cy7 while bound anti-STAT3 was detected using Cy5-conjugated anti-rabbit IgG. One hundred thousand events were acquired using a LSR II or FACS Calibur flow cytometer (BD Biosciences). Data analysis was performed using WinMDI software. Gates were set on live lymphocytes based on their forward- and side-scatter profiles. Cells stained by an antibody of interest was determined by comparing with cells identically treated with a fluorochrome conjugated matched isotype control antibody.

RESULTS

$CD23^{hi}$ $CD58^{+}$ B cells emerged despite efforts to inhibit IL6 function.

Preliminary experiments suggested that $CD23^{hi}$ $CD58^{+}$ cells were unable to proliferate in the absence of non-proliferating sub-populations. Since one of the two newly emerged sub-populations expressed IL6, a B cell growth factor, we examined whether inhibition of IL6 had an effect on the emergence and proliferation of $CD23^{hi}$ $CD58^{+}$ cells. Experiments were performed using primary cells from three healthy individuals enriched for B cells by depletion of CD3 cells. Neutralizing antibody to IL6, IL6R, or to both was added to CD3-depleted cells 1 hour prior to infection with EBV. Simultaneously, cells were infected with EBV in the presence of identical concentrations of isotype-matched antibodies as control. Figure 1 shows that the presence of neutralizing antibodies at a concentration of 1 μg/ml to IL6, IL6R, or both had no effect on the emergence of $CD23^{hi}$ cells as compared to control cells not incubated with neutralizing antibodies on day 4 following exposure of cells to EBV. Similarly, no effect was observed when cells were harvested on day 6, suggesting continued proliferation of $CD23^{hi}$ cells despite the presence of neutralizing antibodies (data not shown). Antibody concentrations of 5, 10, and 100 μg/ml were also used with similar results (data not shown). Thus, functional inhibition of IL6 and/or IL6R did not demonstrate a negative effect on the emergence or proliferation of $CD23^{hi}$ cells.

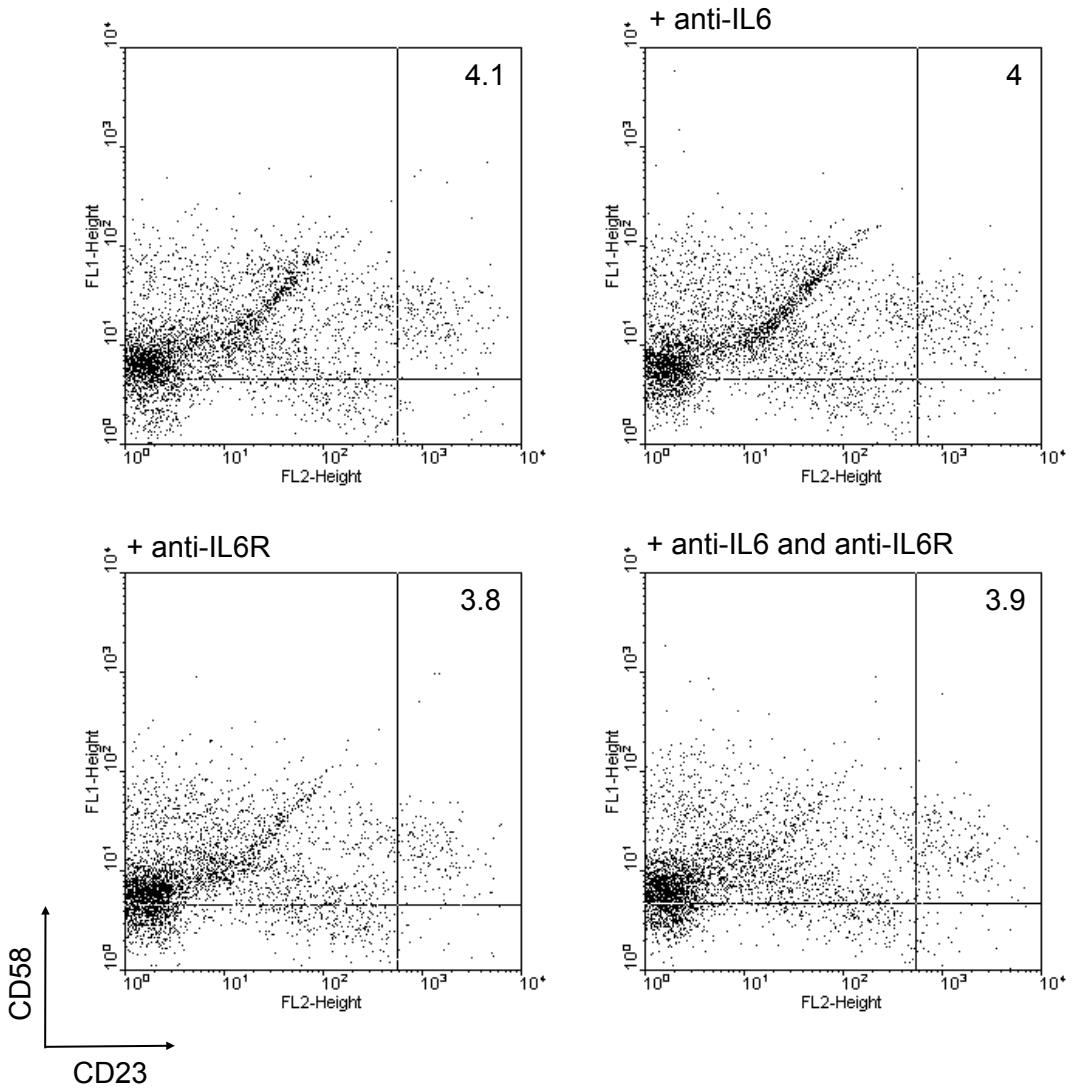

Figure 1. Blocking IL6 has no effect on the emergence of CD23^{hi} CD58^{+} B cells. CD3-depleted cells from EBV-seropositive subject 1 were incubated with anti-IL6, anti-IL6R, or a combination of the two antibodies and exposed to EBV followed by examination of cells stained with antibodies directed against CD58 (FITC) and CD23 (PE) on day 4. Percentages represent fractions of CD23^{hi} CD58^{+} cells of the total.

Correlation between expression of STAT3 and CD23 in B cells exposed to EBV.

Since ligand binding to IL6R triggers activation of the JAK-STAT3 pathway (62), we next examined the effects of EBV infection on levels of STAT3 protein. Interaction of IL6 with IL6R results in activation of STAT3 to phospho-STAT3 (P-STAT3 at residue Y705) via JAK tyrosine kinases. Following dimerization and nuclear translocation, P-STAT3 transcriptionally activates *Stat3* in addition to a myriad of other target genes. Findings in the Bhaduri-McIntosh lab revealed that exposure of B cells to EBV is followed by phosphorylation of STAT3 at Y705 and that LCL have high levels of total STAT3 (Megyola *et al.*, unpublished data). Therefore, using total STAT3 as an indirect measure of phosphorylation of STAT3, we sought to correlate levels of expression of STAT3 with CD23 expression and with emergence of $CD23^{hi}$ cells after exposure of B cells to EBV. Figure 2 shows that when B cells were cultured alone for 4 days, 12% of the total B cell population expressed high levels of STAT3 ($STAT3^{hi}$). As expected, no $CD23^{hi}$ cells emerged. In contrast, when B cells were exposed to EBV for 4 days, 41.3% of cells were $STAT3^{hi}$. Furthermore, 4.3% of the total cells were $CD23^{hi}$ and co-expressed high levels of STAT3. Whereas $CD23^{lo}$ cells could express high or low levels of STAT3, most $CD23^{hi}$ cells expressed high levels of STAT3. Therefore, exposure to EBV resulted in an increase in the fraction of B cells expressing high levels of total STAT3. In addition, $CD23^{hi}$ cells demonstrated expression of high levels of STAT3.

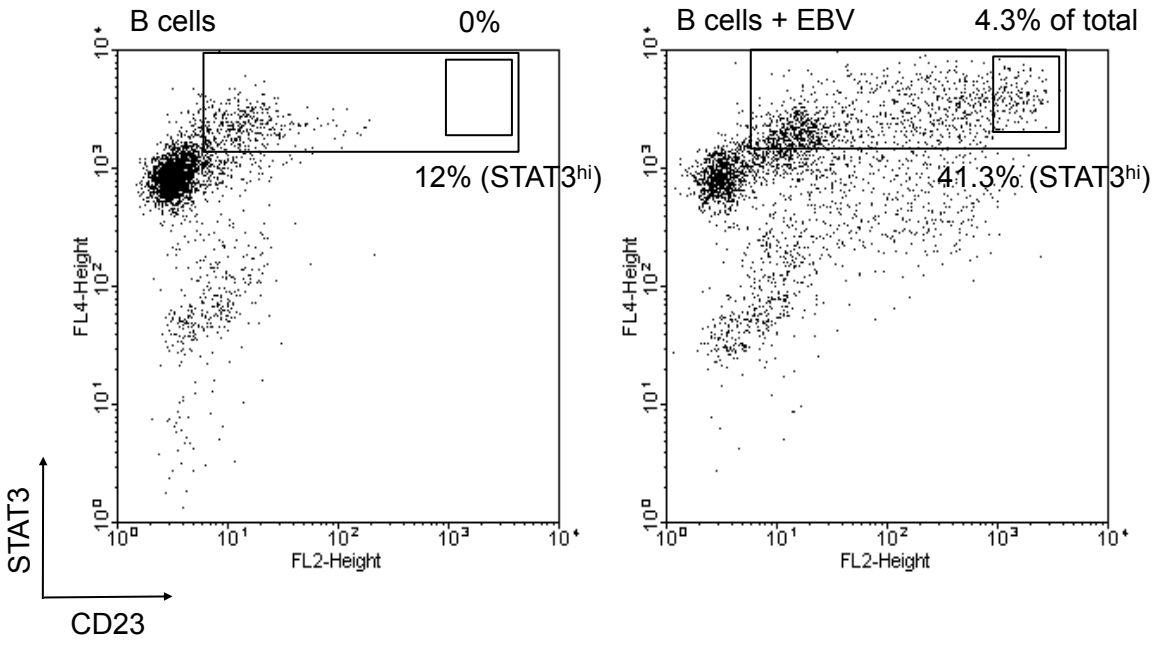

Figure 2. Correlation between expression of STAT3 and CD23 in B cells exposed to EBV. CD3-depleted cells from EBV-seropositive subject 2 were exposed to EBV followed by examination of cells stained with antibodies directed against STAT3 (Cy5) and CD23 (PE) on day 4. Percentages represent fractions of $STAT3^{hi}$ and $STAT3^{hi}$ $CD23^{hi}$ cells of the total.

Inhibition of STAT3 with Stattic abrogates the emergence of all three CD23$^+$ B cell populations and results in near-complete inhibition of STAT3 expression.

Previous experiments in the Bhaduri-McIntosh lab revealed that exposure of B cells to EBV is followed by phosphorylation of STAT3 at Y705. In addition, my experiments (Fig.2) showed that CD23^{hi} cells express high levels of STAT3. We therefore asked if STAT3 is necessary for the emergence of CD23^{hi} CD58$^+$ B cells that are predicted to proliferate following EBV infection. While constitutively active STAT3 was required for LMP1-mediated promotion of tumors in an LMP1 transgenic immunocompromised mouse tumor model (66), the requirement for STAT3 in an infection model may not be obligatory. To address this question, we infected CD3-depleted cells from 2 healthy EBV-seropositive adults with EBV in the presence of Stattic, a small molecule STAT3 inhibitor that interferes with STAT3 activation, dimerization, and nuclear translocation (75). Three different concentrations of Stattic were tested. We compared the fractions of CD23^{hi} CD58$^+$, CD23^{lo} CD58$^+$, and CD23^{lo} CD58$^-$ cells and examined expression of STAT3 on day 4 after exposure of B cells to EBV in the presence of different concentrations of Stattic or solvent as control. While Figure 3 shows data from subject 3, a range of values for both subjects is included in the text. Even at the lowest concentration of 10μM Stattic, all three CD23$^+$ populations failed to emerge as compared to solvent (DMSO) control (Fig.3A). While STAT3$^+$ cells were 81.8% (range: 60.5-81.8) of the total when cells were infected in the presence of DMSO, only 5.9% (3.6-5.9) of cells were STAT3$^+$ when cells were infected in the presence of 10μM Stattic (Fig.3B). Thus, Stattic inhibited nearly all STAT3 expression at the lowest concentration tested.

Trypan blue dye exclusion was used to determine if Stattic was toxic to cells. Table 1 shows that even at 10μM, the lowest concentration tested, Stattic resulted in the death of nearly 81% of cells as compared to cells incubated with DMSO. The negative effect on cell viability was more pronounced at higher concentrations of Stattic. Therefore, Stattic appeared to have a global effect on cells exposed to EBV that included inhibition of emergence of all $CD23^+$ sub-populations, inhibition of expression of STAT3, and cell viability. While the presence of DMSO did not adversely affect cell viability (data not shown), substantial cell death after exposure of B cells to EBV (DMSO condition in Table 1) was not unexpected.

	Condition	Number of cells
Number of cells seeded in culture on day 1		6×10^6
Number of cells harvested on day 4 following exposure to EBV in the presence of	DMSO	8×10^5
	10μM Stattic	1.5×10^5
	20μM Stattic	1×10^5
	50μM Stattic	2.5×10^4

Table 1. Subject 3 Stattic Trypan blue exclusion assay.
The total numbers of EBV-exposed B cells seeded in culture on day 1 and harvested on day 4 in the presence of different concentrations of Stattic or solvent are shown.

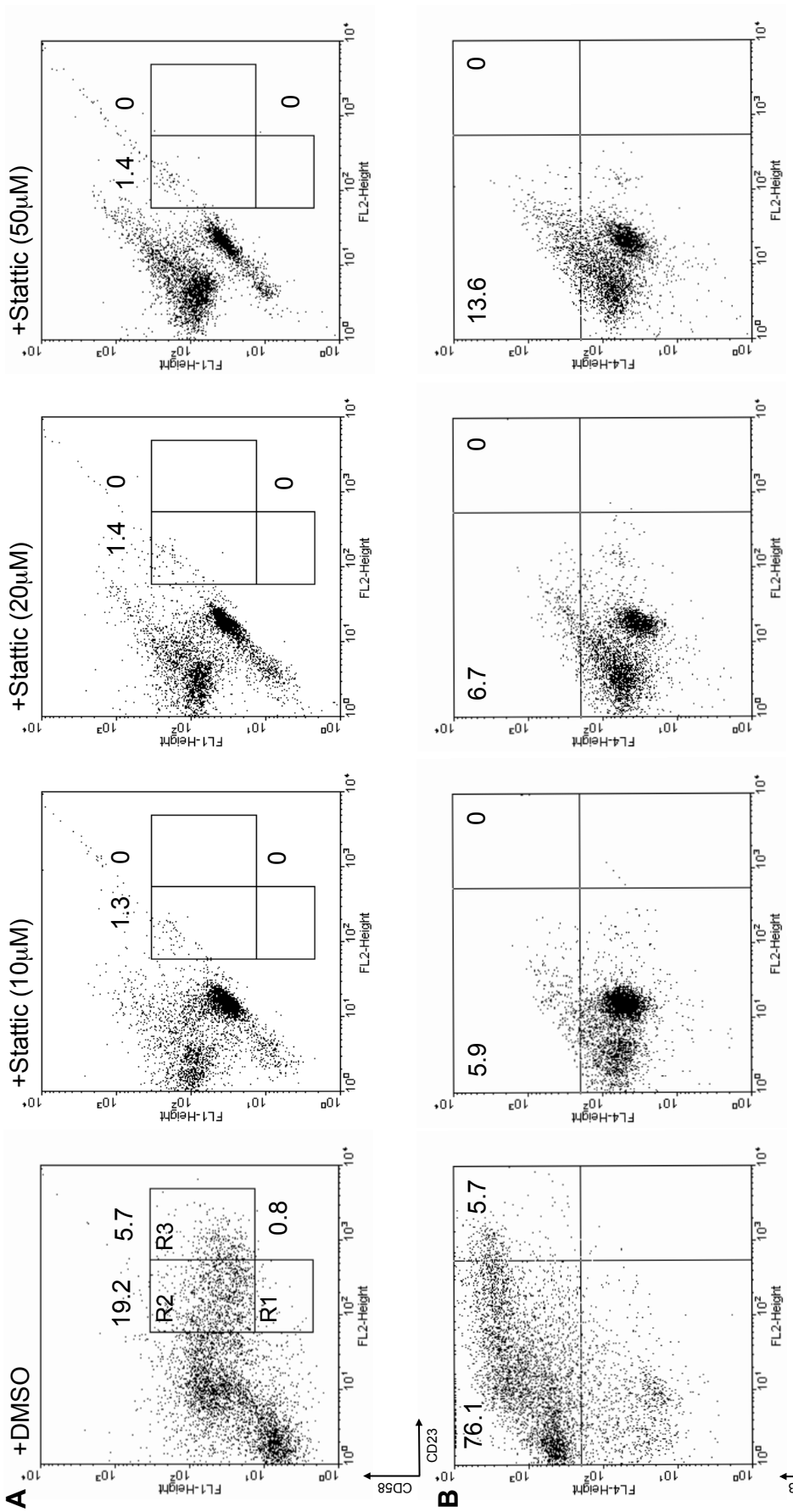

Figure 3. Inhibition of STAT3 with Stattic abrogates the emergence of all three CD23$^+$ B cell populations and results in near-complete inhibition of STAT3 expression. CD3-depleted cells from EBV-seropositive subject 3 were simultaneously exposed to different concentrations of Stattic and EBV followed by examination of cells stained with antibodies directed against A. CD58 (FITC) and CD23 (PE), and B. STAT3 (Cy5) and CD23 (PE) on day 4. Regions R1, R2, and R3 represent CD23^{lo} CD58$^-$, CD23^{lo} CD58$^+$, and CD23^{hi} CD58$^+$, respectively. Percentages represent fraction of cells in each region.

AG490 preferentially inhibits emergence of $CD23^{hi}$ $CD58^{+}$ B cells and results in a fall in the $STAT3^{hi}$ population.

Since Stattic was toxic to EBV-exposed B cells, we decided to test AG490, an inhibitor that specifically blocks phosphorylation of STAT3 at Y705 by inhibiting JAK2 (74). Table 2 shows that AG490 was not toxic to cells at any of the three concentrations tested. Figure 4 and 5 show data from subject 3 using AG490. There was a 5.7-fold fall in the emergence of $CD23^{hi}$ $CD58^{+}$ cells and a 3.1-fold fall in the emergence of $CD23^{lo}$ $CD58^{+}$ cells in the presence of 5μM AG490 (Fig.4A). When 25μM AG490 was used, there was a 14.3-fold fall in the emergence of $CD23^{hi}$ $CD58^{+}$ cells compared to a 4.7-fold fall in the emergence of $CD23^{lo}$ $CD58^{+}$ cells. At a concentration of 50μM, AG490 resulted in a 190-fold fall in the emergence of $CD23^{hi}$ $CD58^{+}$ cells with a 10.1-fold reduction in $CD23^{lo}$ $CD58^{+}$ cells. There were minimal differences in the percentages of $CD23^{lo}$ $CD58^{-}$ cells. Thus, AG490 preferentially inhibited the emergence of $CD23^{hi}$ $CD58^{+}$ cells.

The presence of AG490 did not affect the percentage of $STAT3^{+}$ cells (Fig.4B). Figure 5 shows that if STAT3 expression was divided into low, intermediate, and high levels, no fall in the fraction of cells expressing low levels of STAT3 was observed at any concentration of AG490. There was no fall at 5μM but a 1.2-fold fall at 25μM, and a 1.9-fold fall at 50μM in the percentage of cells expressing intermediate levels of STAT3. In comparison, there was a 2.8-fold, an 8.4-fold, and a 15-fold fall in the percentage of cells expressing high levels of STAT3 in the presence of 5μM, 25μM, and 50μM AG490, respectively. Thus, AG490 demonstrated preferential inhibition of the $STAT3^{hi}$

population. AG490 did not affect IL6 expression at any of the concentrations tested (data not shown).

	Condition	Number of cells
Number of cells seeded in culture on day 1		6×10^6
Number of cells harvested on day 4 following exposure to EBV in the presence of	DMSO	8×10^5
	5μM AG490	1.6×10^6
	25μM AG490	8×10^5
	50μM AG490	1.2×10^6

Table 2. Subject 3 AG490 Trypan blue exclusion assay. The total numbers of EBV-exposed B cells seeded in culture on day 1 and harvested on day 4 in the presence of different concentrations of AG490 or solvent are shown.

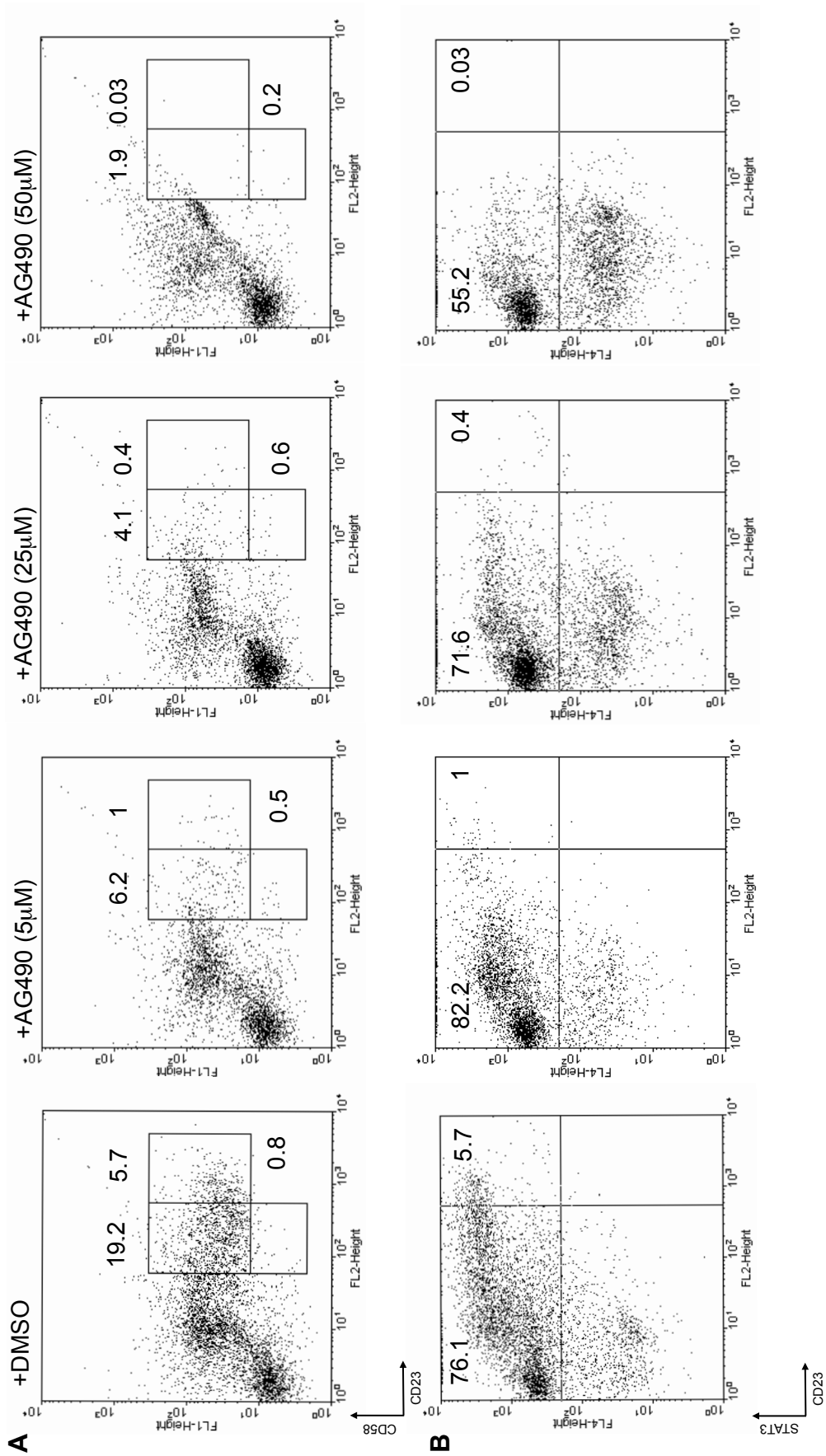

Figure 4. AG490 preferentially inhibits emergence of CD23^{hi} CD58^{+} B cells. CD3-depleted cells from EBV-seropositive subject 3 were incubated with different concentrations of AG490 and exposed to EBV followed by examination of cells stained with antibodies directed against A. CD58 (FITC) and CD23 (PE), and B. STAT3 (Cy5) and CD23 (PE) on day 4. Percentages represent fraction of cells in each region.

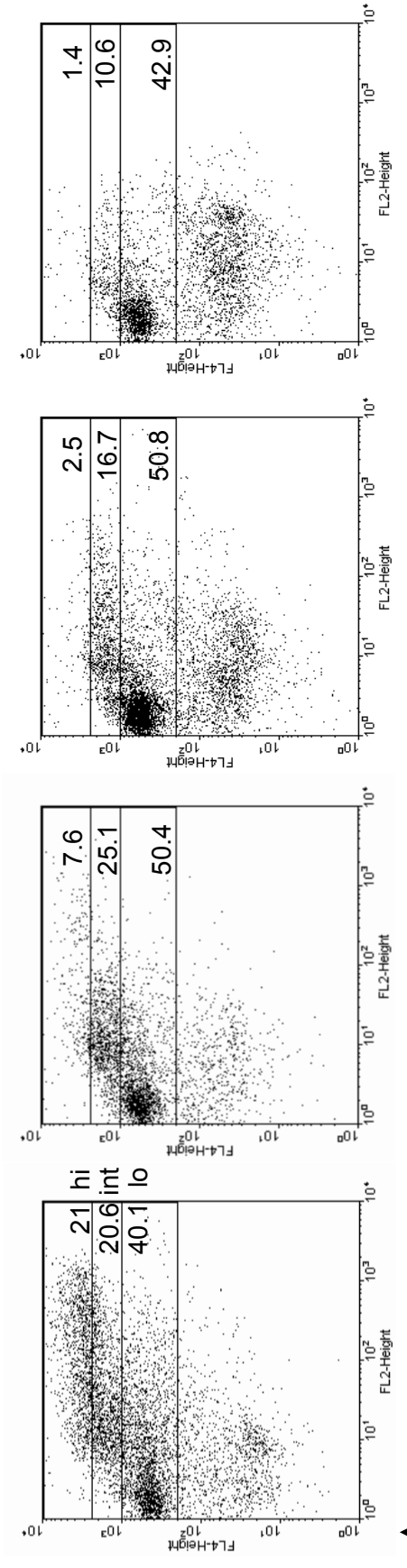

Figure 5. AG490 results in a fall in the STAT3^{hi} population. CD3-depleted cells from EBV-seropositive subject 3 were incubated with different concentrations of AG490 and exposed to EBV followed by examination of cells stained with antibodies directed against STAT3 (Cy5) and CD23 (PE) on day 4. For data analysis, STAT3^{+} cells were divided into STAT3^{hi}, STAT3^{int}, and STAT3^{lo} based on levels of expression of STAT3. Percentages represent fraction of cells in each region.

The effect of AG490 on the emergence of cells predicted to proliferate is observed during the first 24 hours of exposure of B cells to EBV.

We then investigated the window of time during which AG490 could negatively affect the emergence of $CD23^{hi}$ $CD58^{+}$ cells. We exposed CD3-depleted cells from a healthy EBV-seropositive adult in the presence of 25μM AG490. AG490 was added at different times (0-72 hours) following exposure of cells to EBV, and all cells were harvested at 96 hours post exposure. Figure 6 shows that when AG490 was added from 0-24 hours after exposure to EBV, the fraction of $CD23^{hi}$ $CD58^{+}$ cells decreased to 0.3-1% of total as compared to 5.2% in the control group (+EBV+DMSO), resulting in a 5.2- to 17.3-fold reduction. The negative effect on the emergence of $CD23^{hi}$ $CD58^{+}$ cells was lost when AG490 was added after 24 hours. As was observed in earlier experiments, the effect on emergence of $CD23^{lo}$ $CD58^{+}$ cells was less substantial (Fig.4A) with 1.3-2.5-fold reduction when AG490 was added from 0 to 24 hours after exposure to EBV. Results of experiments in Figure 6 suggest that phosphorylation of STAT3 during the first 24 hours of infection with EBV is important for the emergence of B cells predicted to proliferate.

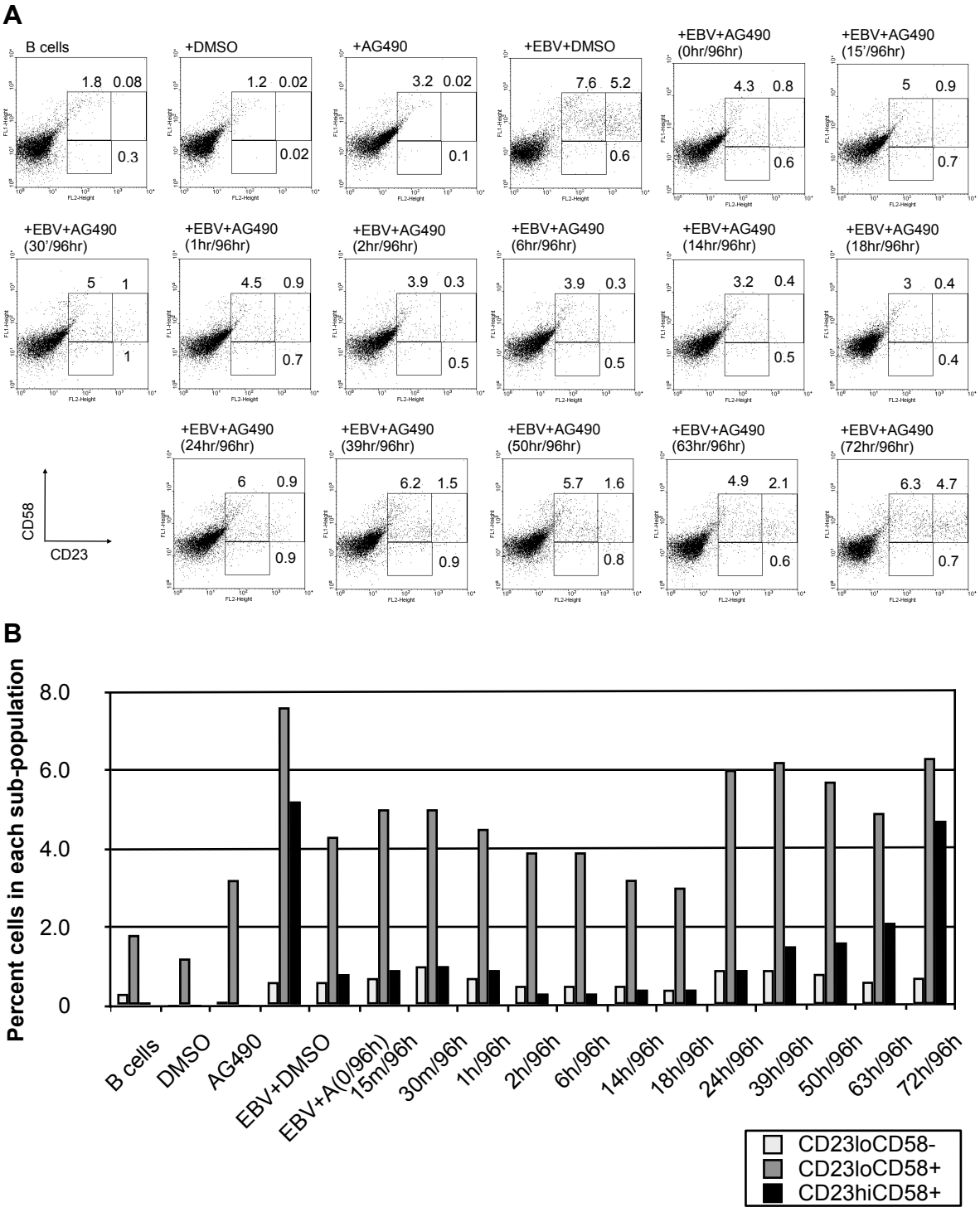

Figure 6. The effect of AG490 on the emergence of cells predicted to proliferate is observed during the first 24 hours of exposure of B cells to EBV. CD3-depleted cells from EBV-seropositive subject 2 were exposed to EBV in the presence of 25μM AG490 added at different times after addition of EBV. Cells were washed after 2 hours of incubation with EBV; AG490 was maintained in culture for the duration of the experiment. Cells were harvested at 96 hours after exposure to EBV. Cells were stained with antibodies directed against CD58 (FITC) and CD23 (PE). Percentages represent fraction of cells in each region. Raw data is shown in A and a graphic representation is shown in B.

DISCUSSION

Since EBV-driven B cell outgrowth has been commonly studied by examining B cell lines 3-8 weeks after infection with EBV (79, 80), many unanswered questions surround the early stages following EBV infection. It is an event central to the persistence of the virus and the development of EBV-associated B cell lymphomas in immunocompromised individuals, in whom a decline in effective T cell surveillance can lead to uncontrolled EBV-driven lymphoproliferation. Given the rise of infectious and iatrogenic causes of immunosuppression and the absence of a vaccine or specific therapy directed against EBV, an ubiquitous virus, a better understanding of early events that lead to proliferation of B cells upon infection with EBV is critical. These lines of investigation will contribute to our knowledge of the pathogenesis of EBV lymphomas and likely lead to promising alternate modes of prevention and therapy.

Although CD23 is generally believed to be an early marker for immortalization (4), findings in the Bhaduri-McIntosh lab demonstrate that EBV differentially infects B cells into distinct sub-populations, only one of which, those marked $CD23^{hi}$ $CD58^{+}$ $IL6^{-}$, can be predicted to proliferate as early as 3 days after exposure to EBV (Megyola *et al*., submitted manuscript). Another sub-population of cells was found to produce IL6 but did not proliferate. As IL6 promotes B cell growth, these findings raise the possibility that non-proliferating sub-populations share the common goal of potentiating proliferation of a select few, possibly via IL6. They also found that expression of high levels of STAT3 correlated with maintenance of the latent state (67). These findings, in conjunction with evidence linking IL6 (37, 40, 45, 46, 47, 48, 49, 50) and STAT3 (60, 63) to EBV

lymphomagenesis, prompted us to investigate the role of both in driving proliferation of newly infected primary B cells.

The experiments presented address questions regarding the role of IL6 and STAT3 in the emergence of B cells predicted to proliferate after exposure of B cells to EBV. Blocking IL6 did not abrogate the emergence of $CD23^{hi}$ $CD58^{+}$ cells as was our initial hypothesis. Consistent with prior findings in the lab, STAT3 expression increased, and $CD23^{hi}$ cells emerged after exposure to EBV. We subsequently showed that inhibiting STAT3 activity with Stattic abrogated the emergence of all three $CD23^{+}$ B cell populations and knocked out nearly all STAT3 expression. AG490, on the other hand, had a preferential negative effect on $CD23^{hi}$ $CD58^{+}$ B cells and resulted in a decrease in the $STAT3^{hi}$ and $STAT3^{hi}$ $CD23^{hi}$ populations. This enabled us to determine that phosphorylation of STAT3 is important for the emergence of cells marked $CD23^{hi}$ $CD58^{+}$ and predicted to proliferate, especially within the first 24 hours after EBV infection. However, this study is not without its limitations.

Constitutively active STAT3 has been detected in LCL (63) and in B cell- and epithelial cell-derived EBV tumors (60, 63). Our lab previously observed a high level of expression of STAT3 in LCL but not in uninfected, cultured B cells. Additionally, experiments revealed that phosphorylation of STAT3 followed exposure of B cells to EBV (Megyola *et al*., unpublished data). While this study adds to what is known about STAT3 in EBV tumorigenesis, what is not clear in the data presented here is the importance of high levels of STAT3 in B cell proliferation given that AG490 is known to target STAT3 phosphorylation via its specific inhibition of JAK2 in the JAK-STAT3

signal transduction pathway. Downregulation of STAT3 expression using siRNA is an option, as well, but current approaches do not deliver siRNAs into a high percentage of primary cells without cytotoxicity (83), resulting in low transfection efficiency. To conclusively demonstrate the importance of high levels of STAT3 in the emergence of $CD23^{hi}$ $CD58^{+}$ cells, the same experiments will need to be performed using primary B cells from individuals with the hyper-IgE or Job's syndrome, a rare disorder of immunity and connective tissue predominantly caused by mutations in *Stat3*, most of which are located in the SH2 and DNA binding regions. Production of proteins with dominant negative activity results in the characteristic symptoms of dermatitis, boils, cyst-forming pneumonias, elevated serum IgE levels, retained primary dentition, and bone abnormalities (84).

While we found that 10-20.8% of $STAT3^{hi}$ cells were also $CD23^{hi}$ and thus predicted to proliferate, it is interesting to note that a substantial number of $CD23^{lo}$ cells were also $STAT3^{hi}$ but did not proliferate. It is possible that STAT3, athough expressed at high levels, may not be phosphorylated in this sub-population of B cells. Alternative mechanisms of transcribing *Stat3* gene beside P-STAT3-mediated transcription may exist. This possibility is supported by the observation that inhibition of $CD23^{lo}$ $CD58^{+}$ sub-population is relatively and that of $CD23^{lo}$ $CD58^{-}$ sub-population is completely spared in the presence of AG490. Another plausible explanation is that inhibitors of STAT3 phosphorylation such as the SOCS family of JAK inhibitors and PIAS (60) may be induced specifically in $CD23^{lo}$ cells.

In our use of Stattic and AG490, we must also consider any possible pleiotropic inhibitory effects pointing to STAT3-independent B cell proliferation. Specific and selective STAT3 inhibitors are few in number. Many compounds have multiple targets or are not specific to STAT3 (85). AG490, a specific Jak2 inhibitor, may interrupt other signal transduction pathways that rely on phosphorylation by Jak2 besides that of STAT3. Comparing the levels of expression of the anti-apoptotic and pro-proliferative molecules Bcl-2, Bcl-xL, Mcl-1, and c-Myc (56, 61), which are transcriptional targets of P-STAT3, by immunoblot using specific antibodies would allow one to directly assess the downstream effects of P-STAT3. Expression of transcriptional targets of unphosphorylated STAT3 such as IL8, ICAM1, and RANTES (55) would indicate if unphosphorylated STAT3 is involved in the early processes that follow exposure of B cells to EBV.

Our data also shows that phosphorylation of STAT3 is only important for emergence of $CD23^{hi}$ $CD58^{+}$ cells during the first 24 hours after infection with EBV, which begs the question of why this effect disappears past 24 hours. It is possible that viral proteins made 24 hours following EBV infection compensate for the lack of STAT3 phosphorylation and proceed to take over the task of maintaining B cell proliferation. A candidate viral protein in this case might be LMP-1. Its key role in EBV-induced proliferative transformation is suggested by evidence such as its ability to transform rodent fibroblasts and increase tumorigenicity in nude mice (34). Furthermore, its expression following EBV infection was not observed earlier than 48 hours (86). While LMP-1 has also been shown to activate STAT3 via IL6-dependent and IL6-independent

mechanisms (65, 87), whether this involves STAT3 phosphorylation is unclear. It is possible that LMP-1 activates expression of STAT3 in the absence of an effect on phosphorylation of STAT3 in B cells.

Although blocking IL6 and IL6R showed no effect on emergence of $CD23^{hi}$ $CD58^{+}$ cells in our experiment, the possibility that IL6 plays a role in emergence of $CD23^{hi}$ $CD58^{+}$ cells cannot be excluded. IL6 has been found to be a growth factor for LCL in culture (40) and in SCID mice (50). The strong correlation between the incidence of post-transplant EBV lymphomas and markedly increased levels of serum IL6 (88) cannot be ignored. The potential role of IL6 in aiding the cells destined to proliferate is also strongly suggested by death of cells when the sub-populations were sorted and reintroduced into culture separately (Megyola *et al*., unpublished data).

Initially, we had considered that we may not have used a high enough concentration of the neutralizing antibodies. Earlier studies with anti-IL6 antibody showed that biological activity of IL6 was not inhibited at 1:1 molar ratio of IL-6:anti-IL-6 antibody (89). However, this is unlikely in this case as a concentration of 100 μg/ml of anti-IL6 was used, which is much higher than reported elsewhere. Complete blockade of IL6 may be necessary to see an effect on the emergence of $CD23^{hi}$ $CD58^{+}$ cells. It is possible that even small amounts of IL6 may be sufficient for the emergence of proliferating cells to proceed. An alternative approach is to remove IL6-producing $CD23^{lo}$ cells after infection by sorting and then compare outgrowth of $CD23^{hi}$ cells in the presence of neutralizing antibodies to IL6 and IL6R with that of mock sorted cultures.

We also considered that the affinity of the neutralizing antibodies for their targets was sub-optimal or that the antibodies had an opposite, excitatory, effect. Given this plausible explanation and the time constraints within which we were working, we decided to focus our investigation on STAT3 rather than identify alternate sources of reagents. Ideally, however, one would repeat the experiments with perhaps a different batch of neutralizing antibodies.

Overall, these experiments imply that our hypothesis regarding STAT3 was correct and encourage further experiments to shed light on the early events in EBV-driven B cell proliferation and the interactions between IL6, STAT3, B cells, and EBV. The hope is that this will enable us to identify and to institute early interventions in EBV lymphoma prevention and innovative therapeutic strategies. Before this can occur, however, we need a more well-founded and complete understanding of these interactions, which will further our comprehension of the underlying pathogenesis of EBV lymphomas.

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