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Anti-TNFα Therapy for Inflammatory Bowel Diseases is Associated With Epstein-Barr Virus Lytic Activation

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Abstract

Anti-TNFα therapy, known to suppress T-cell immunity, is increasingly gaining popularity for treatment of autoimmune diseases including inflammatory bowel diseases (IBD). T-cell suppression increases the risk of B-cell EBV-lymphoproliferative diseases and lymphomas. Since EBV-lytic activation is essential for development of EBV-lymphomas and there have been reports of EBV-lymphomas in patients treated with anti-TNFα therapy, we investigated if patients treated with anti-TNFα antibodies demonstrate greater EBV-lytic activity in blood. Peripheral blood mononuclear cells from 10 IBD patients *solely* on anti-TNFα therapy compared to 3 control groups (10 IBD patients not on immunosuppressive therapy, 10 patients with abdominal pain but without IBD, and 10 healthy subjects) were examined for the percentage of T-cells, EBV load and EBV-lytic transcripts. Patients on anti-TNFα therapy had significantly fewer T-cells, greater EBV load, and increased levels of transcripts from EBV-lytic genes of all kinetic classes compared to controls. Furthermore, exposure of EBV-infected B-cell lines to anti-TNFα antibodies resulted in increased levels of *BZLF1* mRNA; *BZLF1* encodes for ZEBRA, the viral latency-to-lytic cycle switch. Thus, IBD patients treated with anti-TNFα antibodies have greater EBV loads likely due to enhanced EBV-lytic gene expression and anti-TNFα antibodies may be sufficient to activate the EBV lytic cycle. Findings from this pilot study lay the groundwork for additional scientific and

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AUTHOR CONTRIBUTIONS

S.L. and S.B.-M. designed the research; S.L., S.K., and S.S. performed the experiments; S.L. and S.B.-M. analyzed the data and wrote the manuscript; and R.R. and A.C. provided access to study subjects.

clinical investigation into the effects of anti-TNFα therapy on the life cycle of EBV, a ubiquitous oncovirus that causes lymphomas in the setting of immunocompromise.

Keywords

inflammatory bowel disease; anti-TNFα; Epstein-Barr virus; lytic activation; lymphomas

INTRODUCTION

Epstein-Barr virus (EBV), a ubiquitous γ-herpesvirus found in greater than 90% of the adult population [Sankaran-Walters et al., 2011], is associated with 1% of tumors worldwide [Michelow et al., 2012; Dowd et al., 2013]. EBV is etiologically linked to several malignancies including nasopharyngeal carcinoma, Burkitt lymphoma, and various forms of B-cell lymphomas including Hodgkin and non-Hodgkin lymphomas [Crawford, 2001]. The most common type of EBV-lymphomas in the western world occurs in the setting of T cell-immunocompromise, most often after hematopoietic or solid organ transplantation [Gottschalk et al., 2005]. The risk of such lymphomas is also increased in patients with autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases (IBD) who are treated with T cell-immunosuppressive/immunomodulatory therapies [Kamel et al., 1993; Dayharsh et al., 2002; Sokol et al., 2012]. While immunomodulatory agents such as methotrexate, azathiopurine and 6-mercaptopurine have been the mainstay of therapy for autoimmune diseases, anti-TNFα antibodies are now being used with increasing frequency [Kozuch and Hanauer, 2008].

Use of anti-TNFα antibodies alone or in combination with other immunomodulatory agents has been found to be associated with an increased risk of non-Hodgkin lymphomas in IBD patients compared to patients treated with immunomodulatory agents alone [Siegel et al., 2009; Herrinton et al., 2011]. While several cases of EBV-lymphomas following anti-TNFα therapy have been described [Komatsuda et al., 2008; Park et al., 2008; Mariette et al., 2010], whether treatment with anti-TNFα antibodies is associated with an increased risk of EBV-lymphomas is unclear, particularly since several of these patients were simultaneously treated with other immunomodulatory agents. Furthermore, evidence for increase in blood EBV-load following anti-TNFα therapy is conflicting [Lavagna et al., 2007; Fernandez Salazar et al., 2013; Magro et al., 2013], and its contribution to EBV lytic activation, if any, is unknown.

Similar to other herpesviruses, EBV can exist in a latent or lytic state [Knipe and Howley, 2013]. Periodically, EBV undergoes lytic activation when the majority of viral genes including the immediate early, early and late lytic genes are expressed in a specific kinetic order to replicate the viral genome and produce infectious virus [Knipe and Howley, 2013]. This switch from latency to lytic cycle is mediated by two immediate-early genes, *BZLF1* and *BRLF1* which code for the transcriptional activating proteins, ZEBRA and RTA, respectively. Activation of this viral lytic replication pathway is important for the development of EBV-related malignancies. Indeed lytic gene expression was seen in 92% of biopsies of EBV-lymphoproliferative diseases/lymphomas in immunosuppressed patients

[Montone et al., 1996]. Furthermore, methotrexate, a medication frequently used to treat patients with rheumatoid arthritis, was shown to promote EBV-positive lymphomas by its immunosuppressive properties as well as by activating lytic cycle of EBV [Feng et al., 2004]. Moreover, mice with severe combined immunodeficiency did not develop EBV-lymphoproliferative diseases/lymphomas if EBV lytic gene activation was defective [Hong et al., 2005], underscoring the importance of EBV lytic activation for development of EBV-lymphomas during immunocompromise.

In this pilot study we examined the relationship between anti-TNFα therapy and EBV lytic activation in blood cells of patients with IBD treated with anti-TNFα antibodies alone, i.e., in the absence of other immunomodulatory agents. We report higher EBV loads and increased EBV lytic gene expression in peripheral blood cells of IBD patients on anti-TNFα therapy compared to untreated IBD patients and two other control groups. We also demonstrate that anti-TNFα antibodies are sufficient to induce the EBV lytic cycle in vitro.

METHODS

Study Subjects

The study of human subjects was approved by the appropriate Institutional Review Board at Stony Brook University. A total of 40 EBV-seropositive subjects (positive for IgG antibodies to Epstein-Barr Nuclear Antigen and Viral Capsid Antigen) ranging in age from 13 years to 62 years were recruited if they fell into one of the four following categories: no medical problems (10 subjects), abdominal pain but found via upper endoscopy and colonoscopy not to have IBD (10 subjects), IBD who had never been on immunosuppressive agents (10 subjects), IBD solely on infliximab for 6 or more months (10 subjects). Patients with concurrent diagnoses unrelated to IBD were excluded from the study.

PBMC Isolation

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation from 30–35 ml of heparinized venous blood using lymphocyte separation medium (Ficoll-Hypaque; ICN) according to a standardized protocol [Bhaduri-McIntosh et al., 2008]. PBMC were counted and subjected to flow cytometry, qPCR and qRT-PCR.

Culture Conditions

EBV-infected B cell lines (lymphoblastoid cell lines) were generated from healthy subjects as described previously [Hui-Yuen et al., 2011]. Cells were cultured at 37°C under 5% CO_2 in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin sodium, and 100 μg/ml streptomycin sulfate. For the experiment using anti-TNFα antibodies, cells were sub-cultured, treated with 8 μg/ml (equivalent to therapeutic doses in patients) anti-TNFα antibodies (InvivoGen) or control human IgG1 24 hr later, and harvested for qRT-PCR after another 24 hr.

Flow Cytometry

PBMC were subjected to flow cytometry as previously described [Bhaduri-McIntosh et al., 2008]. Briefly, cells were fixed and permeabilized using Cytofix/Cytoperm (BD

Pharmingen, San Diego, CA) and then incubated with saturating amounts of either APC-conjugated anti-CD2 antibody (BD Pharmingen) or matched isotype control antibody. Samples were acquired using FACSCalibur (BD) and data analyzed using Flow Jo software (Treestar Inc, Ashland, OR), after gating on live cells based on forward scatter and side scatter values. Cells were determined to be $CD2^+$ after comparing with isotype control-stained cells.

Quantitative PCR

DNA was extracted from PBMC using the QIAamp DNA blood kit (Qiagen) according to manufacturer's instructions. EBV load was quantified based on a standard curve PCR generated using plasmid 2089 [Feederle et al., 2000] and primers designed to amplify the *BALF5* gene; standard qPCR curve gave linear detection over 5 logs of target concentrations. Plasmid 2089 comprises the B95.8 EBV genome cloned into an F-factor plasmid. Primer sequences to amplify *BALF5* were as follows: forward primer 5′CGGAAGCCCTCTGGACTTC3′; reverse primer 5′CCCTGTTTATCCGATGGAATG3′.

Quantitative RT-PCR

Total RNA was extracted from PBMC using the RNeasy kit (Qiagen) followed by DNase digestion (Promega) and cDNA synthesis as described previously [Hill et al., 2013]. Levels of lytic gene transcripts were determined by real-time reverse transcriptase-PCR (RT-PCR) with gene-specific primers using the iScript SYBR green RT-PCR kit (Bio-Rad). Relative transcript levels were calculated using the ΔΔCT method after normalization to 18S rRNA. Assays on individual samples were performed in triplicate. Primer sequences for *18S, BMRF1* and *BFRF3* were described previously [Hill et al., 2013]. Sequences of other primers were as follows: *BZLF1*: forward primer 5′TTCCACAGCCTGCACCAGTG3′; reverse primer 5′GGCAGCAGCCACCTCACGGT3′; *BRLF1*: forward primer 5′ACTCCCGGCTGTAAATTC CT3′; reverse primer 5′CCATACAGGACACAACACCT CA3′.

Statistical Analyses

P values were calculated by comparing the means of two groups of interest using unpaired Student *t* test.

Ethical Considerations

Informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

RESULTS

Characteristics of IBD Patients and Control Subjects

Our goal was to investigate EBV load and lytic activation in the blood of patients treated with anti-TNFα antibodies alone. We therefore recruited IBD patients treated with anti-TNFα antibodies without the confounding presence of immunomodulatory agents. Furthermore, we only recruited patients who had been on anti-TNFα therapy for at least 6

months. Table I shows that compared to control groups, patients on anti-TNFα therapy had a preponderance of males. The mean ages were similar except for IBD control subjects who were younger ($P \leq 0.01$). Both groups with IBD were similar with regard to type of disease (Crohn's Disease [CD] versus Ulcerative Colitis [UC]). While IBD controls had carried their diagnosis for a shorter period of time, by an average of 13 months ($P \leq 0.03$), the number of IBD patients in remission was the same in both groups and none had severe disease activity. IBD patients on infliximab (anti-TNFα antibodies) had been on this medication for a mean duration of 28 ± 12 months.

Since infliximab has been shown to suppress T cell activation and proliferation [Dahlen et al., 2013], we enumerated the frequency of peripheral T and NK cells as a general assessment of cellular immune status. As expected, flow cytometry of immunostained PBMC revealed that IBD patients on infliximab had an average of 16% fewer $CD2^+$ cells compared to the other three groups ($P \leq 0.05$) (Fig. 1). Sub-group analysis of the three patients on infliximab with moderate disease activity showed that these patients had on average only 8.5% fewer $CD2^+$ cells compared to the control subjects. This is consistent with the observation that reduced frequencies of activated T cells correspond with treatment response to anti-TNFα antibodies [Dahlen et al., 2013]. There was also significant improvement in the erythrocyte sedimentation rate following treatment with anti-TNFα antibodies; however, the corresponding fall in C - reactive protein was not statistically significant.

IBD Patients Treated With Anti-TNFα Antibodies Demonstrate Higher EBV Load

To determine EBV load in PBMC, we amplified the EBV DNA polymerase gene *BALF5* using qPCR. Although EBV genomes were detected in all subjects recruited in the study, IBD patients on anti-TNFα antibodies had on average 578 genomes (range 116–1341 genomes) per 10^6 PBMC as compared to the other three groups which collectively had on average 161 genomes (range 5–430 genomes) per 10^6 PBMC (Fig. 2). This corresponded to a > 3.5-fold increase in viral load in the anti-TNFα group, a statistically significant increase compared to the EBV load in each of the three control groups. The three patients with moderate disease activity on infliximab had an average of 386 genomes per 10^6 PBMC which was below the study group average but still greater than the control groups.

IBD Patients on Anti-TNFα Antibodies Demonstrate Higher Levels of EBV Lytic Gene Transcripts Belonging to All Kinetic Classes

As IBD patients on anti-TNFα antibodies revealed greater EBV load, we asked whether this increase in EBV genomes stemmed from increased EBV lytic activation. We therefore compared relative levels of EBV lytic gene transcripts amongst the four study groups by amplifying mRNA derived from the EBV immediate early lytic genes *BZLF1* and *BRLF1*, early lytic gene *BMRF1*, and late lytic gene *BFRF3*. These genes were chosen as representatives of the three kinetic classes of EBV lytic genes. We found that on average, IBD patients on anti-TNFα antibodies had greater than 5.5-fold increase in *BZLF1* and *BRLF1* transcript levels compared to the three control groups ($P \leq 0.01$) (Fig. 3A–B). *BZLF1* and *BRLF1*, encoding for transcription factors ZEBRA and RTA respectively, mediate EBV latency-to-lytic switch [Knipe and Howley, 2013]. Transcript levels of *BMRF1* and *BFRF3*

were on average sixfold and threefold higher, respectively in the anti-TNFα group compared to the other study groups ($P \leq 0.04$) (Fig. 3C–D). The early lytic gene *BMRF1,* which is transcriptionally activated by ZEBRA, encodes for the DNA polymerase processivity factor (Early Antigen-Diffuse) and is essential for EBV replication [Neuhierl and Delecluse, 2006]. *BFRF3* is a late lytic gene encoding a structural capsid protein which aids in packaging EBV DNA leading to the release of infectious EBV particles [Serio et al., 1997]. Thus, in addition to demonstrating higher EBV loads, patients on anti-TNFα also demonstrated higher levels of EBV lytic transcripts.

Anti-TNFα Antibodies Activate the Lytic Molecular Switch in B Cells Latently Infected With EBV

Increased lytic gene expression could result from impaired T cell surveillance or anti-TNFα-mediated lytic cycle activation in EBV-infected B cells. To address if anti-TNFα antibodies are capable of activating the EBV lytic cycle, we exposed previously established EBV-infected B cell lines to anti-TNFα antibodies and measured transcript levels of *BZLF1*, the critical molecular lytic switch. As shown in Figure 4, addition of anti-TNFα antibodies resulted in significantly higher levels of *BZLF1* transcripts compared to control in both cell lines, indicating that anti-TNFα antibodies are sufficient to activate the EBV latency-to-lytic cycle switch.

DISCUSSION

In this study, we report that treatment with anti-TNFα antibodies is associated with increased EBV load in peripheral blood cells, this increase is at least partially a result of increased EBV lytic cycle activation and anti-TNFα antibodies are sufficient to activate the EBV latency-to-lytic switch. While anti-TNFα antibody-mediated suppression of immune surveillance may also contribute to EBV lytic activation, this remains to be determined. Although a pilot study using a small number of subjects, to our knowledge, this is the first to examine whether anti-TNFα antibodies are associated with a shift in the balance between EBV latency and lytic cycles.

Evidence for increased EBV loads in autoimmune patients treated with TNFα blockers is conflicting. A few studies found substantial fractions of patients while others reported a minority of patients treated with anti-TNFα antibodies to have higher EBV loads [Reijasse et al., 2004; Lavagna et al., 2007; Magro et al., 2013]. A prominent confounding factor in these studies was concurrent treatment with immunomodulating agents. Therefore, our primary recruitment criterion was to include patients with IBD who were treated solely with anti-TNFα without concomitant immunomodulator therapy. Because there are very few such patients, we were only able to recruit 10 within the time constraints that we had. We also chose to include 1) IBD patients lacking immunomodulatory therapy and 2) patients with abdominal pain without IBD to control for the possibility of IBD causing EBV lytic cycle activation. While these factors also contributed to the small sample size of our study, nevertheless, we felt that it was important to have clearly-defined study and control groups to aid in defining the contribution of TNFα-antagonists to EBV load and lytic cycle activation.

In assessing the demographics of our study subjects, we found that there were gender differences between the four groups (Table I). This was partly due to difficulty identifying IBD patients solely on anti-TNFα therapy as well as locating IBD control subjects not on any immunomodulatory treatment. Since there is no known association between gender and EBV load or development of EBV-lymphomas, the differences in gender distribution are likely not to be confounding factors. The mean ages were similar except for IBD control subjects who were younger ($P \leq 0.01$). This age range is in line with the natural epidemiology of IBD in which most patients are diagnosed in adolescence and childhood [Gasparetto and Guariso, 2013]. Furthermore, the shorter duration of disease in IBD controls was expected as anti-TNFα therapy is typically reserved for refractory IBD because of safety concerns. Adverse effects of anti-TNFα antibodies have included fatal cases of tuberculosis, hepatitis B virus reactivation, and increased risk of cancer [Lavagna et al., 2007]. However of note, the fraction of patients in remission was identical in both the IBD groups. It is also important to point out that the EBV loads calculated for healthy controls in our study are similar to those observed by Balandraud et al. who reported copy numbers ranging from 0–43.7 per 1.5×10^5 PBMC [Balandraud et al., 2003]. Furthermore, suppression in the T and NK cell fraction is consistent with apoptosis of T cells (as well as macrophages) that has been demonstrated in IBD patients on anti-TNFα antibodies [ten Hove et al., 2002; Van den Brande et al., 2003; Di Sabatino et al., 2004]. In summary, our results indicate that any future study with a prospective study design would need to recruit IBD patients at initial diagnosis and obtain laboratory information on multiple parameters including EBV copy number, T cell number, cellular or viral markers and EBV serologic status before and after commencement of anti-TNFα therapy.

Most lymphomas in IBD patients on immunosuppressive therapy are of B-cell origin with almost 50% being positive for EBV [Sokol et al., 2012; Fernandez Salazar et al., 2013]. Also, IBD patients treated with anti-TNFα ±immunomodulators were found to have a higher risk for NHL compared to those only on immunomodulators [Siegel et al., 2009]. However, how many of these tumors were EBV-positive is not known. Indeed, the risk of development of non-Hodgkin lymphomas is high when EBV load exceeds 5,000 copies/10^6 mononuclear cells [Reijasse et al., 2004]. What fraction of this load is derived from replication of latent EBV genomes versus replication of lytic genomes which can yield 100–1000-fold amplification of the genome in a linear form [Serio et al., 1997], is unclear. Our discovery of EBV lytic cycle activation including increased transcripts from a late lytic gene indicates that increased EBV loads in patients on anti-TNFα therapy were derived from lytic EBV genomes. While none of the subjects in our study had EBV loads greater than 1,341 copies/10^6 mononuclear cells, our study had a relatively short follow-up period. Our finding that anti-TNFα therapy is associated with a shift towards EBV lytic activation, despite the small study group, provides the basis for larger prospective studies on patients treated with anti-TNFα therapies.

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Abbreviations

IBD	inflammatory bowel disease
EBV	Epstein-Barr virus
CD	crohn's disease
UC	ulcerative colitis

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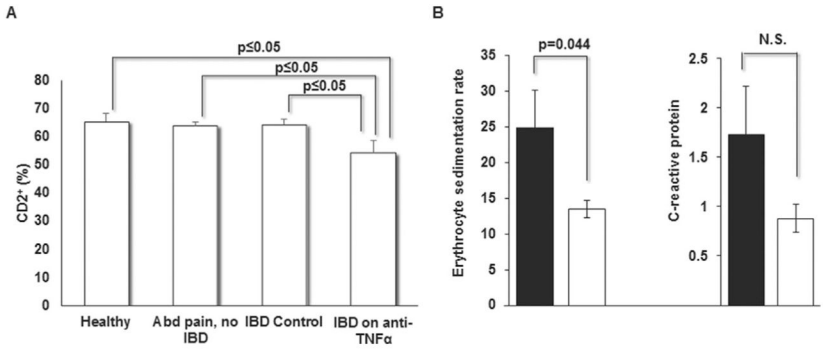

Fig. 1.

IBD patients on anti-TNFα have fewer T and NK cells and improvement in inflammatory markers. PBMC from patients and control subjects were immunostained and $CD2^+$ cells enumerated by FACS in **A**. Inflammatory markers, erythrocyte sedimentation rate and c-reactive protein, before (black bars) and after (open bars) anti-TNFα therapy are shown in **B**. Data represent mean +/− SEM.

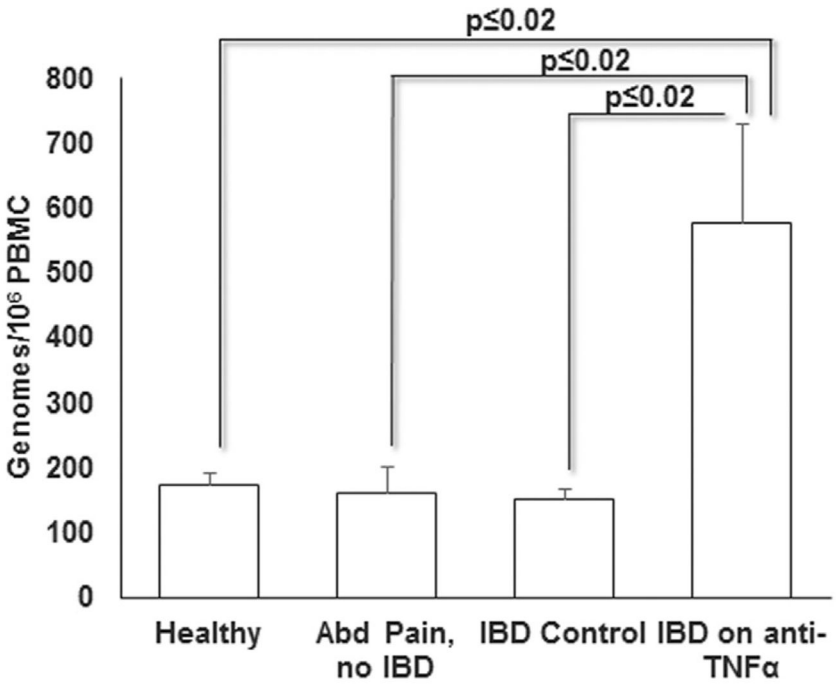

Fig. 2.

IBD patients on anti-TNFα antibodies demonstrate greater EBV load. EBV load (mean genome copies +/− SEM) per 10^6 PBMC was determined in patients and control subjects by qPCR using the standard curve method.

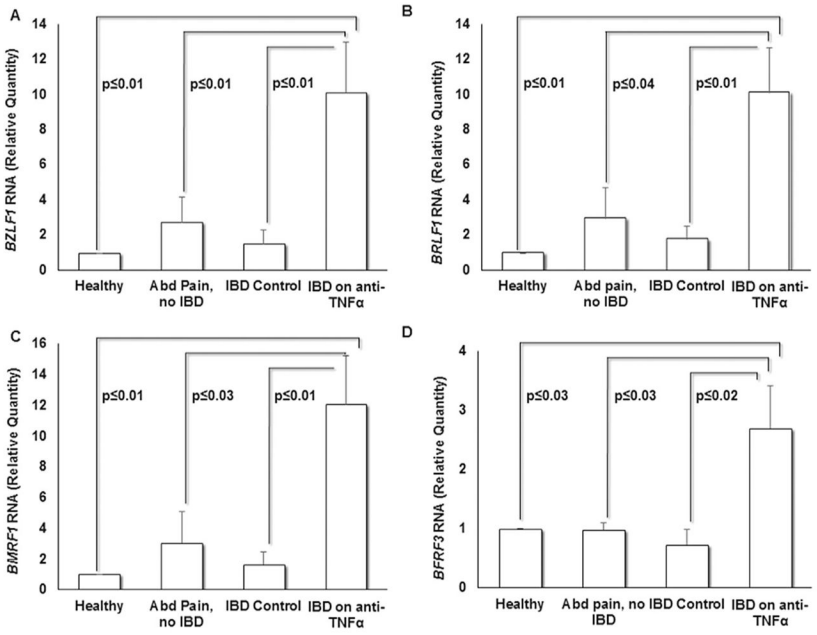

Fig. 3.

Greater EBV lytic gene expression is seen in IBD patients on anti-TNFα antibodies. PBMC were subjected to qRT-PCR using primers targeting immediate early lytic genes *BZLF1* (**A**) and *BRLF1* (**B**), the early lytic gene *BMRF1* (**C**) and the late lytic gene *BFRF3* (**D**). Results represent means of relative amounts of RNA normalized to 18S rRNA levels +/− standard errors of the means of three technical replicates.

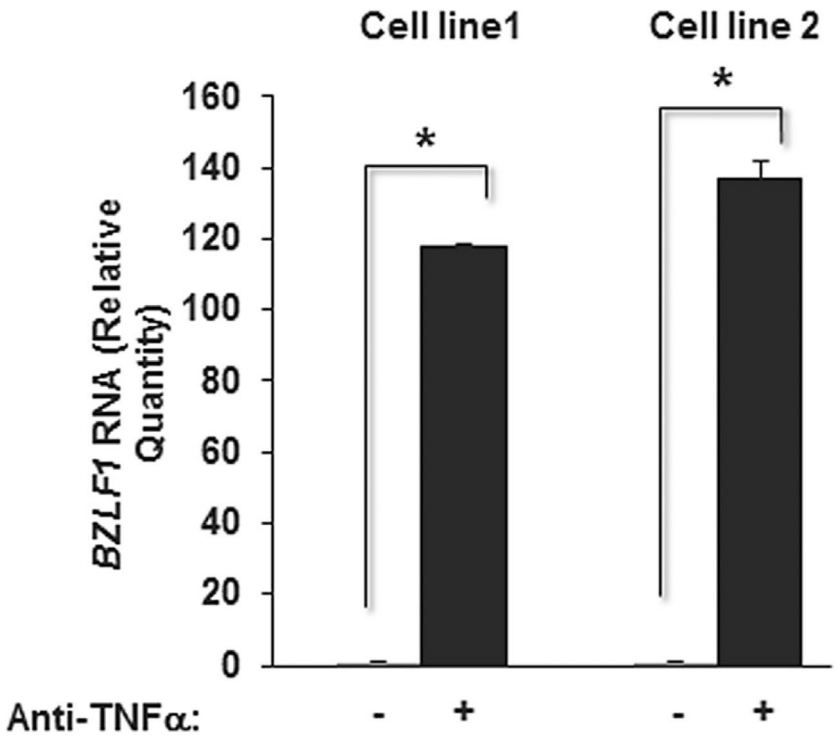

Fig. 4.

Anti-TNFα antibodies induce the EBV lytic switch in latently infected B cells. EBV-infected B cell lines were treated with anti-TNFα antibodies (or control human IgG1 antibody), harvested after 24 hr and subjected to qRT-PCR using primers targeting transcripts from the EBV latency-to-lytic cycle switch gene *BZLF1*. Results represent means of relative amounts of RNA normalized to 18S rRNA levels +/− standard errors of the means of three technical replicates.

TABLE I

Characteristics of Study Subjects

	Healthy (n = 10)	Abd pain, no IBD (n = 10)	IBD control (n = 10)	IBD on anti-TNFα (n = 10)
Sex				
Male	1	4	6	8
Female	9	6	4	2
Age (Mean ± SD)	34 ± 8 years	26 ± 13 years	23 ± 8 years	33 ± 13 years
Types of IBD				
CD	----------		6	4
UC			4	6
Duration of disease (Average months ± SD)	----------		23 ± 12	36 ± 12
Severity of disease*	----------		Remission 6 Mild 3 Moderate 1	Remission 6 Mild 1 Moderate 3
Duration of anti-TNFα use	----------			28 ± 12 months

CD, Crohn's Disease; UC, ulcerative colitis.

*Measured by Physician's Global Assessment.