ORIGINAl ARTICLE

IL-15 prolongs CD154 expression on human CD4 T cells via STAT5 binding to the CD154 transcriptional promoter

RM Lowe1,2, A Genin1, N Orgun3 and RQ Cron1,2

Activation-induced CD154 expression on CD4 T cells is prolonged in systemic lupus erythematosus, but the mechanism(s) for its dysregulation are unknown. The studies reported herein demonstrate that interleukin-15 (IL-15) is capable of prolonging CD154 expression on phytohemagglutinin (PHA)-activated CD4 T cells. As IL-15 signals through signal transducer and activator of transcription 5 (STAT5), predicted STAT5 binding sites in the human CD154 transcriptional promoter were identified, and STAT5 binding to the proximal CD154 promoter in vitro and in vivo following primary CD4 T-cell activation was demonstrated. Moreover, overexpression of wild-type STAT5 in primary human CD4 T cells augmented CD154 transcription, whereas overexpression of a dominant-negative (DN) STAT5 protein inhibited CD154 transcription. Mutation of the most proximal STAT5 binding site in the CD154 promoter resulted in diminished DNA binding and reduced CD154 transcriptional activity. Interestingly, STAT5-specific small interfering RNA inhibited CD154 surface expression at 48 but not 24 h after T-cell activation. Thus, these findings provide some of the first evidence to support a possible mechanistic link to explain how the overexpression of IL-15 observed in lupus patients may be involved in the prolonged expression of CD154 that has also been observed on lupus CD4 T cells.

Genes and Immunity (2014) 15, 137–144; doi:10.1038/gene.2014.3; published online 6 February 2014

Keywords: human; T cells; CD154; IL-15; STAT5; lupus

INTRODUCTION

CD154 (CD40 ligand) is a 39-kDa protein transiently expressed on the surface of activated CD4 T cells.1 Normally, CD154 expression is strictly controlled, predominantly at the level of transcription.1 However, abnormal expression of CD154 has been linked to the pathology of several autoimmune diseases, particularly systemic lupus erythematosus (SLE).2 In prior studies, we have demonstrated that lupus patients have elevated and prolonged expression of CD154 on CD4 T cells.2 In a study by Yi et al.,3 cyclosporin A was found to reduce CD154 expression by inhibiting the NFAT-calcineurin pathway. However, in the same study, cyclosporin A was observed to lose potency after approximately 18 h following T-cell activation, suggesting that other transcription factors also contribute to prolonged expression.3 In a different study authored by Baranda et al.,4 lupus patients were observed to have elevated serum interleukin-15 (IL-15) levels. From prior studies by other groups, there is published data demonstrating that IL-15 binding to its receptor on T cells triggers Jak 1 and Jak 3 to phosphorylate tyrosine residues on signal transducer and activator of transcription 5 (STAT5).5,6 Upon activation, STAT5 translocates to the nucleus and binds the γ-interferon-activated sequence (GAS), defined by the TTCN5GAA consensus sequence.7 This event ultimately leads to increased transcription of target genes. These known pathways and the independently observed associations of the aberrant expression of CD154 on T cells in lupus patients and elevated serum levels of IL-15 in lupus patients in independent studies prompted us to further explore whether CD154 expression may be regulated directly by STAT5.

Our prior work with CD154 expression and interest in understanding the potential mechanisms to help identify and characterize dysregulated signaling pathways in lupus prompted us to search for potential binding sites (BS) for transcription factors that may bind to the CD154 transcriptional promoter and influence its expression. Our sequence analysis of the CD154 promoter resulted in the identification of three potential BS for STAT5 within and just upstream of the CD154 promoter (Figure 1). This suggests that STAT5 may have a role in regulating CD154 transcription. Although many cytokines are capable of signaling via STAT5, IL-2, in particular, has been shown to be decreased in lupus blood.8 By contrast, another related cytokine that signals via STAT5, IL-15, has been shown to be aberrantly overexpressed in lupus.9–11 and may therefore contribute to CD154 hyperexpression via STAT5 signaling, especially at later time points after T-cell activation.12 To further characterize one of the potential mechanisms by which IL-15 may influence CD154 expression through STAT5, we performed the studies described within this manuscript using CD4 T cells obtained from healthy individuals. Herein, we provide clear evidence of how STAT5 has a direct role in CD154 expression in primary CD4 T cells.

RESULTS

Stimulation by IL-15 and PHA leads to persistent upregulation of CD154

In exploring a role for IL-15 and STAT5 in CD154 regulation, the first question addressed was whether IL-15 induces or augments the expression of CD154 in primary CD4 T cells. Human peripheral

1Department of Pediatrics, University of Alabama at Birmingham School of Medicine, Birmingham, AL, USA; 2Division of Rheumatology, The Children’s Hospital of Alabama, Birmingham, AL, USA and 3Fred Hutchinson Cancer Research Center, Seattle, WA, USA. Correspondence: Dr R Lowe, Department of Pediatrics, University of Alabama at Birmingham/Children’s of Alabama, 1600 7th Avenue South, Children’s Park Place, Suite M210, Birmingham, AL 35233-1711, USA.
E-mail: rlowe@peds.uab.edu
Received 30 May 2013; revised 29 November 2013; accepted 23 December 2013; published online 6 February 2014
blood CD4 T cells isolated by negative selection were stimulated ex vivo with phytohemagglutinin (PHA), IL-15 or a combination of both for 6, 20 or 48 h. Flow cytometry data indicating CD154 surface expression levels are shown (Figure 2). Low-dose PHA or IL-15 alone did not increase CD154 expression significantly, but stimulation of the primary CD4 T cells with both PHA and IL-15 led to notable CD154 expression. This CD154 expression continued to increase throughout the later time points following activation. These data support the hypothesis that IL-15 significantly augments CD154 transcription in stimulated CD4 T cells, particularly beyond the first 24 h of stimulation. Further experiments were designed to characterize whether this effect is mediated at least in part through STAT5, a known mediator of IL-15 signaling. Subsequent experiments were performed to identify BS of STAT5 in the human CD154 promoter.

STAT5 binds two sites in the human CD154 promoter in vitro. A search of the Transfac database immediately upstream of the human CD154 transcriptional start site (TSS) yielded three potential STAT5 BS using the consensus binding sequence, TTCNGAA: BS no. 1 (−57 to −65 bp); BS no. 2 (−298 to −306 bp); and BS no. 3 (−2807 to −2815 bp) (Figure 1). To verify experimentally whether the STAT5 transcription factor binds to these sites in vitro, nuclear extracts were generated from primary human CD4 T cells stimulated with PHA and IL-2, a cytokine known to induce STAT5 expression. These extracts were probed with radiolabeled oligonucleotides containing the putative STAT5 BS sequences (BS nos. 1–3) from the human CD154 promoter. Autoradiographs showing the results of electrophoretic mobility shift assays (EMSA) are shown (Figure 3, left and right panels). No detectable shifted band was seen with the BS no. 2 probe (data not shown). However, the radiolabeled putative STAT5 BS no. 1 or BS no. 3 probes mixed with nuclear extracts formed visible gel-retarded complexes (Figure 3, lane 4, left panel—BS no. 1, right panel—BS no. 3) that migrated an identical distance as the one formed by STAT5 binding to the prolactin (PRL)-positive control probe (Figure 3, both panels, lane 1). Addition of excess unlabeled self or PRL oligonucleotides (Figure 3, left panel,

Figure 1. Consensus STAT5 BS in the proximal human CD154 transcriptional promoter. Three potential BS within 3 kb upstream of the CD154 TSS (arrow) were identified by sequence analysis via the Transfac database and labeled as BS nos. 1–3.

Figure 2. IL-15 augments late CD154 expression on suboptimally stimulated CD4 T cells. Primary human CD4 T cells were rested (red lines) or stimulated (blue lines) with phorbol ester and ionomycin (far left column), low-dose PHA alone (left middle column), PHA plus IL-15 (right middle column) or IL-15 alone (far right column) for 6 (top row), 20 (middle row) or 48 h (bottom row) before analysis of cell surface CD154 expression by flow cytometry. Results are representative of four similar experiments.
lane 5 and data not shown) or BS no. 3 probe (Figure 3, right panel, lanes 5 and 6) before adding labeled BS no. 1 or BS no. 3 probes resulted in the disappearance of this band, providing evidence of sequence-specific competition. The addition of antibody specific for the N-terminal domain of STAT5 causes the complex to supershift (lanes 3 and 7, both panels), indicating that the binding protein to PRL, BS no. 1 and BS no. 3 is STAT5. To further demonstrate specificity of STAT5 binding to the proximal human CD154 promoter, the BS no. 1 sequence was altered by 3 bp, resulting in a BS no. 1-mutant oligonucleotide probe that eliminated its ability to bind to the STAT5 protein (Figure 3, lanes 8–11). Consistent with this observation, an excess of unlabeled BS no. 1-mutant probe (Figure 3, left panel, lane 6) only minimally competed with binding of the labeled, non-mutated BS no. 1 probe (Figure 3, left panel, lane 4). Thus, STAT5 binds the proximal human CD154 promoter BS no. 1 sequence as well as the upstream BS no. 3 sequence in activated primary human CD4 T cells.

STAT5 engagement of the proximal human CD154 promoter in vivo is important for optimal CD154 transcription. Chromatin immunoprecipitation (ChIP) was used to isolate DNA that was specifically bound to the STAT5 transcription factor in vivo following activation of primary CD4 T cells. Peripheral blood human CD4 T cells were stimulated with either phorbol myristate acetate and ionomycin (P + I, positive control), stimulated with PHA and IL-15 or left unstimulated (negative control) before being subjected to ChIP. Real-time polymerase chain reaction (PCR) was performed on anti-STAT5 antibody-immunoprecipitated DNA fragments to demonstrate and quantify the degree of STAT5 binding to the CD154 proximal promoter in vivo. The bar graph (Figure 4) reveals minimal if any specific STAT5 binding in the absence of stimulation (light gray bar) compared with the isotype control antibody (black bar). However, PHA plus IL-15 stimulation (dark gray bar) resulted in a notable increase in the percentage of amplified DNA compared with total input DNA demonstrating STAT5 binding to the proximal CD154 promoter in vivo. This was more marked when the cells were polyclonally stimulated with phorbol ester and calcium ionophore (white bar) such that the mean STAT5 fold-binding (relative to isotype control antibody) to the proximal human CD154 promoter was 16.7-fold (16.7 ± 6.9 s.e.m.; n = 3) in vivo after 24 h incubation of P + I-stimulated primary human T cells. Thus, STAT5 binds the proximal human CD154 promoter in vivo following activation of primary CD4 T cells.
The presence of STAT5 influences transcriptional activity of the CD154 promoter
To determine the functional relevance of STAT5 binding to the proximal CD154 promoter, an ex vivo dual-luciferase reporter assay was used. Negatively selected primary human CD4 T cells were co-transfected with a human CD154 promoter-driven luciferase reporter vector that contains the BS no. 1 STAT5BS, and either a wild-type (WT) STAT5 expression vector or a dominant-negative STAT5 (DN STAT5)\(^\text{16,17}\) expression vector. After culturing for 6 h with P + I, or IL-15, cells were assayed for luciferase expression. IL-15 alone did not appear to augment the transcriptional expression of CD154, as the arbitrary light unit (ALU) values of the IL-15-stimulated samples were similar to controls with media alone (Figure 5). However, luciferase expression in samples stimulated with P + I increased CD154 transcription with increasing doses of WT STAT5 plasmid in a dose-dependent manner. Moreover, transfection with the DN STAT5 plasmid markedly diminished ALU levels compared with controls. This finding suggests that overexpression of WT STAT5 augments CD154 promoter activity via direct binding of STAT5 to the CD154 promoter, although IL-15 stimulation alone is not sufficient to induce CD154 transcription. Interestingly, endogenous STAT5 appears important for CD154 transcriptional activity as supported by the observation that transfection of the DN STAT5 construct inhibited CD154 promoter reporter gene activity. 

Mutation of BS no. 1 decreases transcriptional activity of the CD154 promoter reporter gene
To provide further evidence that STAT5 binding to BS no. 1 is important for CD154 transcriptional promoter activity, we constructed a CD154 luciferase expression plasmid containing a mutation within the BS no. 1 site in the proximal promoter. The original pCD154-Luc plasmid\(^\text{18}\) was modified by site-directed mutagenesis, introducing the same 3 bp mutation as was used in the gel shift experiments with the BS no. 1-mutant oligonucleotide that disrupted STAT5 binding to the human CD154 proximal promoter (Figure 3). Primary human CD4 T cells were transiently co-transfected with a human CD154 transcriptional reporter gene vector, either WT control or BS no. 1 mutant, along with a control Renilla luciferase expression vector to control for transfection efficiency. As expected, the BS no. 1 mutant CD154 luciferase expression plasmid demonstrated a decrease of expression by more than 40% (Figure 6), providing additional functional evidence of the importance and specificity of endogenous STAT5 binding to BS no. 1 of the CD154 promoter to promote CD154 transcription.

siSTAT5 inhibits CD154 expression
The essential role of STAT5 augmenting CD154 promoter transcription was further demonstrated by utilizing small interfering RNA (siRNA) technology. Primary human CD4 T cells isolated from peripheral blood were electroporated with expression constructs containing either a negative control random sequence (siRandom) or STAT5 siRNA sequences (see Materials and methods). Electroporated cells were rested overnight and then stimulated with P + I before analyzing cells for CD154 expression by flow cytometry at 24 and 48 h. No significant changes in the level of CD154 surface expression were observed at 6 (data not shown) or 24 h (Figure 7a, left) after stimulation, as measured by percent CD154-positive cells or CD154 mean fluorescence intensity (13% decreased percent positive; 12% decreased mean fluorescence intensity). However, in samples from 9 of 12 donors, CD4 T cells transfected with the siSTAT5 vector showed significant inhibition (> 50%) of CD154 surface expression at 48 h (Figure 7a, right) following stimulation compared with the control using the means of data from multiple experiments (Figure 7b, 57%
decreased mean fluorescence intensity). By 72 h after stimulation, CD154 expression became too low to determine reliably any differences between cells transfected with siSTAT5 vector and the control vector (data not shown).

To verify that the diminished CD154 surface expression could be attributed to STAT5 siRNA interference with STAT5 mRNA levels, we performed real-time reverse transcription-PCR experiments with similarly prepared donor CD4 T cells at 48 h following stimulation with P+I, or PHA plus IL-15, demonstrating a 60% decrease in STAT5 mRNA levels in the presence of STAT5 siRNA (Figure 7c). Interestingly, the siRNA only inhibited CD154 expression at the later time point, suggesting that STAT5 engagement and upregulation of CD154 occurs later after T-cell activation and may reflect the role of IL-15 in vivo, particularly with regard to CD154 overexpression at later time points, as is seen in ex vivo experiments with T cells from SLE patients (also known to overexpress IL-15 in the blood). Thus, STAT5 engagement of the human CD154 promoter and upregulation of CD154 expression occurs later (~24–48 h) after the initial T-cell stimulation.

To confirm increased IL-15 expression in SLE ex vivo and postactivation in vitro similar to our other assays. IL-15 was elevated in lupus patients (n = 2) compared with age-, gender- and race-matched controls (n = 2), as measured in plasma levels (Figure 8, black bars). Patients also had notably elevated levels of IL-15 in CD4 T-cell culture supernatant after 18 h of incubation, either stimulated with anti-CD3Ab/anti-CD28Ab-coated beads (Figure 8, gray bars) or resting without any stimulation (Figure 8, white bars). Although we do not have sufficient numbers of patient and matched controls at this time to show statistical significance, this observation is consistent with our hypothesis that IL-15 is dysregulated in patients with lupus. Of note, both lupus patients had highly active disease when blood was obtained at the time of initial diagnosis. Interestingly, even though one of the two lupus patients had received high-dose steroids (2 days of oral prednisone and one dose of 1 g of intravenous methylprednisolone), IL-15 plasma levels still correlated with severity of disease at the time of testing in this small sample of patients (data not shown).

**DISCUSSION**

Stimulation of primary human T cells with PHA in the presence of IL-15 results in the upregulation of CD154 that begins at 24 h and persists at 48 h following stimulation, implicating a role for IL-15 in CD154 expression. A novel BS for the STAT5 transcriptional activator was located 56–65 bp upstream of the human CD154 TSS and was experimentally verified through EMSA and ChIP. Functional evidence for STAT5 binding to the CD154 promoter was demonstrated using transcriptional reporter genes and mRNA analysis. The importance of endogenous STAT5 in primary human CD4 T cells to CD154 expression was also demonstrated using siRNA technology to knockdown STAT5 mRNA.

In an effort to determine the relative significance of our findings with regard to STAT5 binding on the CD154 promoter, we conducted a search of the ENCODE data set. Our query affirmed the novelty of our findings. There are only two ENCODE cell lines (GM12878—a B-lymphocyte lymphoblastoid human cell line transformed by Epstein–Barr virus and K562—a myelogenous leukemia human cell line) with transcription factor-binding...
expression without further stimulation. These data further support the hypothesis that elevated levels of IL-15 may have links to increased STAT5 activity and its consequent effects on CD154 expression in T cells. However, we are encouraged to pursue this line of research by data generated by other groups studying the pathogenic mechanisms in other autoimmune diseases, such as rheumatoid arthritis. Mottonen et al. published data showing that IL-15 upregulates the expression of CD154 on synovial fluid T cells.

Future research, in addition to improving our understanding of how IL-15 signaling influences CD154 gene expression in health and disease, will also expand to other potentially important mechanisms of CD154 gene regulation including epigenetic modifiers of expression. These include DNA methylation and its effects on CD154 transcriptional activity, as well as microRNA species that alter CD154 translational efficiency. This will hopefully lead to a further improved understanding of how we may therapeutically manipulate CD154 expression in T cells to create novel therapies for the treatment of lupus and other autoimmune diseases.

MATERIALS AND METHODS
Patients and matched controls for IL-15 studies
Peripheral venous blood was drawn from two SLE patients with lupus nephritis (an African-American 13-year-old female and a Caucasian 13-year-old female) and two matched controls (an African-American 12-year-old female and a Caucasian 13-year-old female). All subjects gave informed assent, and informed consent was given by one of each child’s parents. Both SLE patients had blood samples drawn at the time of diagnosis. At this time, lab tests performed were consistent with highly active lupus: ESR > 100, proteinuria with urine protein to creatinine ratio > 2.0, ANA with a titer of 1:160 or higher and abnormally elevated Smith and/or double-stranded DNA antibodies. One SLE patient did not receive any steroids or treatment before drawing blood; the other SLE patient had received 2 days of oral prednisone and 1 g of methylprednisolone on the day before drawing blood. Institutional review board approval was obtained before recruiting volunteers for the collection of blood samples.

CD4 T-cell isolation and stimulation
Primary human CD4 T cells were isolated from the peripheral blood of healthy adult donors, healthy-matched pediatric donors and patients with lupus by negative selection according to the manufacturer’s protocol (RosetteSep; StemCell Technologies Inc., Vancouver, BC, Canada) as described. Flow cytometry confirmed 96–98% CD4 T-cell purity of the resulting cell populations (data not shown). Cells were cultured for 6 h at 37 °C in 5% CO2 in complete RPMI 1640 medium containing 10% fetal calf serum, 2 μM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin alone, or at least the presence of 10 ng/ml phorbol myristate acetate and 1.5 μM ionomycin (Sigma-Aldrich, St Louis, MO, USA). In the IL-15 and CD154 expression studies, cells were stimulated with 1.5 μg/ml PHA (Sigma-Aldrich), or 10 U/ml IL-2, 75 ng/ml IL-15 alone (R&D Systems, Minneapolis, MN, USA) or a combination of 1.5 μg/ml PHA and 75 ng/ml IL-15. Stimulation time was 6, 24 or 48 h. Cells from pediatric lupus patients and healthy matched controls were cultured for...
IL-15 production either with anti-CD3Ab/anti-CD28Ab-coated beads at a 1:1 bead to cell ratio (Dynabeads Human T-Activator CD3/CD28; Life Technologies Inc., Carlsbad, CA, USA) or without any stimulation for 18 h before culture supernatants were collected and stored at −80 °C until assayed for IL-15 concentration as detailed below.

EMSA

Nuclear proteins from stimulated primary human CD4 T cells were extracted as described previously.27 Synthetic oligonucleotides (IDT, Coralville, IA, USA) were used as ±32P-labeled probes or as unlabelled competitors were as follows: BS no. 1: 5′-CACATTCTCCAGAAGTGTGC-3′ (sense) and 5′-CCACATCTCTCAGAATTTGCT-3′ (antisense); BS no. 2: 5′-GTC ATCTTCTGGAAATCGTG-3′ (sense) and 5′-ACAGTTCTAGAGATGGC-3′ (antisense); BS no. 3: 5′-ATACATTCTTCGAAATCGA-3′ (sense); 5′-CATTAGTTCTCAAGAATGTAT-3′ (antisense). The following oligonucleotide was used as a probe for the mutated BS no. 1 (antisense): 5′-CACATTCTCCACCT AGTGGG-3′. Consensus STAT5 BS are in bold letters. Mutated residues are underlined.

In brief, 2 μg of nuclear protein, 25,000 c.p.m. of ±32P-labeled probe and EMSA binding buffer (500 mM Tris–HCl, pH 7.5, 500 mM KCl; 15 mM MgCl2; 10 mg ml−1 bovine serum albumin; 50% glycerol; 5 mg ml−1 single-strand sperm DNA; 1 μM dithiothreitol) were mixed and incubated for 20 min on ice. For specificity determinations, nuclear protein was reacted with excess (200-fold) unlabeled probe, or anti-STAT5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 30 min before the addition of labeled probe. The mixtures were then electrophoresed on 6% TBE–Tris/borate/EDTA gradient prepored DNA retardation gels (Invitrogen, Carlsbad, CA, USA). Radioactive bands were detected by conventional autoradiography as described previously.18

Site-directed mutagenesis

The WT promoter-driven luciferase reporter plasmid, pCD154-Luc, was changed by introducing a 3 bp mutation in BS no. 1 to match the mutant oligonucleotide that no longer binds STAT5 by gel shift assay (Figure 3). We used commercial reagents (Quickchange Site-Directed Mutagenesis Kit; Agilent Technologies, Santa Clara, CA, USA), and followed the manufacturer’s protocol as described previously.28 Briefly, mutant primers for STAT5 BS no. 1, located approximately 60 bp upstream of the CD154 TSS: 5′-GCCAATTGGCTCTCAGATCTCAGCTG-3′ (sense) and 5′-CTTAGTTCTCAAGAATGTATTT-3′ (antisense) primers, were selected and synthesized. Mutated residues are underlined. Twenty-five nanogram per ml of reporter plasmid was used as a template in PCR with selected mutant primers in the final concentration of 1 μM each, and 1 μl per reaction of PfuThermo DNA polymerase. WT supercoiled DNA was then digested with 1 μl of DpnI restriction enzyme and 50 μl of XLI-Blue Supercompetent cells were transformed with 1 μl of digested DNA. Mutations in plasmid DNA purified from selected colonies were confirmed by DNA sequencing analysis.

Luciferase reporter gene assay and plasmid DNA

Primary human peripheral blood CD4 T cells, 5 × 105 to 105 per electrophoresis

co-culture, were transiently co-transfected by AMAXA (Koeln, Germany) as described previously27 with pCD154-Luc (5 μg) containing a 1.3 kb portion of the human CD154 gene (−1227 to +67). Numbers are given with respect to the TSS),18 PRL-null (0.5 μg); Promega, Madison, WI, USA) and pcDNA control (Sigma-Aldrich) vectors, or expression plasmids pSTAT5b (5 μg).10 (a kind gift from Dr N Selliah, Humigen, Trenton, NJ, USA), or pSTAT5b-CA, a kind gift from Dr M Farrar, University of Minnesota, Minneapolis, MN, USA)25,26. After electroporation and a 2-h rest at 37 °C in a CO2 incubator, CD4 T cells were cultured in vitro with phorbol myristate acetate (25 ng ml−1) and ionomycin (1.5 μM), and with or without IL-2 (100 U ml−1) for 6 h at 37 °C. Cell lysates were generated with the Passive Lysis Buffer (Promega Corporation) as described previously.28

Firefly luciferase reporter activity was determined in duplicate and correlated for transfection efficiency based on Renilla luciferase activity (Dual Luciferase Assay Kit; Promega) using a Lumat LB 9507 luminometer (EE and G Berthold, Bad Wildbad, Germany) as detailed previously.31,32 PRL null, a Renilla luciferase plasmid, was co-transfected for the purpose of data correction. An empty expression vector was co-transfected as a control where appropriate.

ChIP assays

ChIP assay used reagents from Upstate Biotechnology (Lake Placid, NY, USA) with modifications as per the manufacturer’s protocol, as described previously.25,35 Nuclear proteins and chromatin were crosslinked and sonicated, and extracts from one to five million cells were precipitated overnight at 4 °C with 5–10 μg of each test antibody: anti-STAT5b (N-20) rabbit polyclonal, anti-NF-kB p50 (NLS) rabbit polyclonal or their respective IgG isotype control antibody (Santa Cruz Labs, Santa Cruz, CA, USA) and Protein G agarose. DNA extracted from each precipitate was probed by TaqMan real-time PCR (Applied Biosystems, Grand Island, NY, USA) using the following oligonucleotide primers upstream of the human CD154 TSS: 5′-TGTTGGTGAGAAGACTCCAG-3′ and 5′-TGCCACCTTTA CTCAGAGATTGA-3′. Fold differences between specific antibody and isotype control immunoprecipitations were calculated using the formula: 2−ΔΔCt, where ΔCt = CtSample − CtControl.

STAT5 knockdown by siRNA

STAT5 knockdown on CD154 protein expression was studied on primary human CD4 T cells through the use of transient transfection of siRNA35 (Custom SMARTpool; Dharmacon, Thermo Scientific, Lafayette, CO, USA). The siRNA cocktail used was a mixture of four different synthetic sequences targeting STAT5 mRNA (U-005169-11, -12, -13 and -14). A non-targeting siRNA pool (D-001810-10-05; Thermo Scientific) was used as a negative control. Primary human CD4 T cells were transfected according to the manufacturer’s protocol (AMAXA) on program U-024 with 4.2 μg per transfection of either STAT5 siRNA or an equal amount of scrambled sequence siRNA. Transfection efficiency was confirmed using the provided pmaxGFP control plasmid. After transfection, cells were incubated without stimulation overnight, and then cultured in the presence of stimulation reagents, or culture medium alone. CD154 protein surface expression was measured by flow cytometry after 24, 48 and 72 h of stimulation with P-1 (Sigma-Aldrich). STAT5 mRNA was measured at 48 h after stimulation. Data for this assay were only evaluated if the siRNA transfection efficiency was 70% or greater.

Flow cytometry and antibodies

The following mouse anti-human antibodies were purchased (BD Biosciences, San Jose, CA, USA): FITC– or PE-conjugated anti-CD4 (clone SK3), IgG1, μ; anti-CD25 (clone 2A3), IgG1, μ; and anti-CD69 (clone FN50), IgG1, μ. The following corresponding isotype control antibodies were also purchased (BD Biosciences Pharmingen, San Diego, CA, USA): PE-conjugated anti-CD154 (clone TRAP1, mouse IgG1, μ) and PE-conjugated anti-CD4 (clone RM4-5, IgG1, μ). Stained cells were analyzed on a FACScalibur (BD Biosciences) and analyzed on FlowJo software (Tree Star Inc., Ashland, OR, USA) as described previously.

Enzyme-linked immunosorbent assay for IL-15

Plasma and CD4 cell culture supernatants were tested for IL-15 from SLE patients (n = 2) and matched controls (n = 2) using a commercially available human IL-15 ELISA (Biosource, USA) as per the manufacturer’s instructions. All samples were tested in duplicate with the mean value used. The detection threshold for this assay was < 11 pg ml−1. All values below the detection threshold given by the manufacturer was assigned a value of 1 pg ml−1. The coefficient of variation for intra-assay precision was 4–5% and the coefficient of variation for interassay precision was 5–9%, as documented by the manufacturer. Recovery of Hu IL-15 added to serum averaged 81%. The recovery of Hu IL-15 added to tissue culture medium containing 1% fetal bovine serum averaged 107%.

Quantitative real-time reverse transcription-PCR

Total RNA was extracted from CD4 T cells by Trizol reagent (Invitrogen) according to the manufacturer’s protocol; about 2.0 μg of total RNA was reverse transcribed using iScript Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), as per the manufacturer’s instructions. Quantitative real-time reverse transcription-PCR experiments were performed in duplicate using the iQ5 iCycler (Bio-Rad Laboratories). The same CDNA served as template for the genes of interest, and as an internal control, for 18S RNA. Total reaction volume was 25 μl in which 0.5 μl of cDNA and 300–900 pmol of primers were used. A template control was routinely performed in duplicate for each primer pair. Amplification curves were analyzed using iQ-5 Optical System Software (Bio-Rad Laboratories). A melting curve program was run at the end of
REFERENCES
1 Cron RQ, CD154 transcriptional regulation in primary human CD4 T cells. ImmunoRes 2005; 27: 185–202
2 Cron RQ, CD154 and lupus. Pediatr Rheumatol Online J 2003; 1: 172–181.
3 Yi Y, McNerney M, Datta SK. Regulatory defects in cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. J Immunol 2000; 165: 6627–6634.
4 Baranda L, de la Fuente H, Layesca-Espinosa E, Fortaleza-Perez D, Nino-Moreno P, Valencia-Pacheco G et al. IL-15 and IL-15R in leucocytes from patients with systemic lupus erythematosus. Rheumatology (Oxford) 2005; 44: 1507–1513.
5 Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens. Annu Rev Immunol 1999; 17: 19–49.
6 Tagaya Y, Bamford RN, DeFilippis AP, Waldmann TA. IL-15: a pleiotropic cytokine with diverse receptor/signaling pathways whose expression is controlled at multiple levels. Immunity 1996; 4: 329–336.
7 Schmitt-Ney M, Doppler W, Ball RK, Groner B. Beta-casein gene promoter activity is regulated by the hormone-mediated relief of transcriptional repression and a mammary-gland-specific nuclear factor. Mol Cell Biol 1991; 11: 3745–3755.
8 Lieberman LA, Tsokos GC. The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. J Biomed Biotechnol 2010; 2010: 740619.
9 Park YB, Kim DS, Lee WK, Suh CH, Lee SK. Elevated serum interleukin-15 levels in the Janus kinase 3-Stat5 pathway in malignant growth of human T cell leukemia virus type 1-transformed human T cells. J Immunol 2003; 171: 5853–5864.
10 Aringer M, Stumvoll GH, Steiniger G, Koller M, Steiner CW, Hoffer E et al. Serum interleukin-15 is elevated in systemic lupus erythematosus. Rheumatology (Oxford) 2001; 40: 876–881.
11 Clark DN, Markham JL, Sloan CS, Poole BD. Cytokine inhibition as a strategy for treating systemic lupus erythematosus. Clin Immunol 2013; 148: 335–343.
12 Chae DW, Nosaka Y, Strom TB, Maslinski W. Distribution of IL-15 receptor alpha-chains on human peripheral blood mononuclear cells and effect of immuno-suppressive drugs on receptor expression. J Immunol 1996; 157: 2813–2819.
13 Matys V, Kel-Margoulis OV, Fricke E, Liebich I, Land S, Barciszewski J et al. TRANSFAC and its module TRANSPATH: transcriptional gene regulation in eukaryotes. Nucleic Acids Res 2006; 34: D108–D110.
14 DaSilva L, Rui H, Erwin RA, Howard OM, Kirken RA, Malabarba MG et al. Prolactin recruits STAT1, STAT3 and STAT5 independent of conserved receptor tyrosines TYR402, TYR479, TYR515 and TYR580. Mol Cell Endocrinol 1996; 117: 131–140.
15 May R, Welte T, Windegger M, Lechner J, May P, Heinrich PC et al. Selective coupling of STAT factors to the mouse prolactin receptor. Eur J Biochem 1998; 258: 784–793.
16 Selliah N, Zhang M, DeSimone D, Kim H, Brunner M, Ittenbach RF et al. The gamma-cytokine regulated transcription factor, STAT5, increases HIV-1 production in primary CD4 T cells. Virology 2006; 344: 283–291.
17 Ahonen T, Xie J, LeBaron MJ, Zhu J, Nummi M, Alainen K et al. Inhibition of transcription factor Stat5 induces cell death of human prostate cancer cells. J Biol Chem 2003; 278: 27287–27292.
18 Schubert LA, King G, Cron RQ, Lewis DB, Aruffo A, Hollenbaugh D. The human gp39 promoter. Two distinct nuclear factors of activated T cell protein-binding elements contribute independently to transcriptional activation. J Biol Chem 1995; 270: 29624–29627.
19 Koshy M, Berger D, Crow MK. Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes. J Clin Invest 1996; 98: 826–837.
20 Desai-Meha A, Lu L, Ramsey-Goldman R, Datta SK. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. J Clin Invest 1996; 97: 2063–2073.
21 Skov S, Bonyhadi M, Odum N, Ledbetter JA. IL-2 and IL-15 regulate CD154 expression on activated CD4 T cells. J Immunol 2000; 164: 3500–3505.
22 Mottonen M, Isomak P, Luukkainen R, Toivanen J, Punnonen J, Lassila O. Interleukin-15 up-regulates the expression of CD154 on synovial fluid T cells. Immunology 2000; 100: 238–244.
23 Zhang Y, Zhao M, Sawalha AH, Richardson B, Lu Q. Impaired DNA methylation and its mechanisms in CD4+ T cells of systemic lupus erythematosus. J Autoimmun 2013; 41: 82–99.
24 Lu Q, Wu A, Tesmer L, Ray D, Yousif N, Richardson B. Demethylation of CD40LG on the inactive X in T cells from women with lupus. J Immunol 2007; 179: 6352–6358.
25 Chen T, Li Z, Jing T, Zhu W, Ge J, Zheng X et al. MicroRNA-146a regulates the maturation process and pro-inflammatory cytokine secretion by targeting CD40L in oxLDL-stimulated dendritic cells. FEBS Lett 2011; 585: 567–573.
26 Cron RQ, Bort SJ, Wang Y, Brunvand MW, Lewis DB. T cell priming enhances IL-4 gene expression by increasing nuclear factor of activated T cells. J Immunol 1999; 162: 860–870.
27 Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with ‘mini-extracts’, prepared from a small number of cells. Nucleic Acids Res 1989; 17: 6419.
28 Selliah N, Zhang M, White S, Zoltick P, Sawaya BE, Finkel TH et al. FOXp3 inhibits HIV-1 infection of CD4 T-cells via inhibition of LTR transcriptional activity. Virology 2008; 381: 161–167.
29 Burchill MA, Goetz CA, Prlic M, O’Neil JJ, Harmon IR, Bansinger SJ et al. Distinct effects of STAT5 activation on CD4+ and CD8+ T cell homeostasis: development of CD4+CD25+ regulatory T cells versus CD8+ memory T cells. J Immunol 2003; 171: 5853–5864.
30 Kirken RA, Erwin RA, Wang L, Wang Y, Rui H, Farrar WL. Functional uncoupling of the Janus kinase 3-STAT5 pathway in malignant growth of human T cell leukemia virus type 1-transformed human T cells. J Immunol 2000; 165: 5097–5104.
31 Behre G, Smith LT, Tenen DG. Use of a promoterless renilla luciferase vector as an internal control plasmid for transient co-transfection assays of Ras-mediated transcription activation. Biotechniques 1999; 261: 24–26, 28.
32 Cron RQ, Bartz SR, Clausell A, Bort SJ, Klebanoff SJ, Lewis DB. NFAT1 enhances HIV-1 gene expression in primary human CD4 T cells. Clin Immunol 2000; 94: 179–191.
33 Torgerson TR, Genin A, Chen C, Zhang M, Zhou B, Anover-Somke S et al. FOXP3 inhibits activation-induced NFAT2 expression in T cells thereby limiting effector cytokine expression. J Immunol 2009; 183: 907–915.
34 Brunner M, Zhang M, Genin A, Ho IC, Cron RQ. A T-cell-specific CD154 transcriptional enhancer located just upstream of the promoter. Genes Immun 2008; 9: 640–649.
35 Cron RQ, Bandyopadhyay R, Genin A, Brunner M, Kesh GJ, Yin J et al. Early growth response-1 is required for CD154 transcription. J Immunol 2006; 176: 811–818.