Supplemental Information

The Transcription Factors Egr2 and Egr3 Are Essential for the Control of Inflammation and Antigen-Induced Proliferation of B and T Cells

Suling Li, Tizong Miao, Meera Sebastian, Punamdip Bhullar, Emma Ghaffari, Mengya Liu, Alistair L. J. Symonds, and Ping Wang
Supplemental Experimental Procedures

Functional analysis of Treg cells in vitro

CD4+ lymph node (LN) T cells were separated into CD4+CD25+ (Treg) and CD4+CD25- (naïve) T cell populations, with a purity >99%, by cell sorting. Wild type naïve CD4+CD25 responder T cells were plated at 3-5 x 10^4/well in round bottom 96-well plates (0.2 ml) together with an equal number of CD4+CD25+ Treg cells, and irradiated (2000R) T cell-depleted B6 splenocytes as accessory cells, and stimulated with anti-CD3 mAb (1μg/ml) for 72 hours. Before harvest, the cultures were pulsed with [3H]-thymidine for 8 hours.

Supplemental Experimental Procedures

Mice

C57BL/6 mice were used as controls in all experiments. Rag-2−/− mice on a C57BL/6J background were bred in-house and described in our previous report (Zhu et al., 2008). CD2-specific Egr2 transgenic mice were reported previously (Li et al, 2011). All mice were maintained in the Biological Services Unit, Brunel University, and used according to established institutional guidelines under the authority of a UK Home Office project license.

Antibodies and Flow cytometry

Fluorescein isothiocyanate (FITC)-conjugated antibodies to B220, CD4, CD8 and TCRβ; phycoerythrin (PE)-conjugated antibodies to BrdU, CD3, CD4, CD8, CD19, CD25, CD62L, CD69, IgM and NK1.1; PerCP labeled antibody to CD3, NK1.1, CD19 and B220; allophycocyanin (APC)-conjugated antibodies to CD44, CD86, CD3, TCRβ, CD19 and APC-conjugated Annexin V were obtained from BD Biosciences. PE-conjugated pre-loaded CD1 tetramer was from ProImmune, while antibody to CD3 (clone 145-2C11) and CD28 (clone
37.51) for stimulation and 7AAD were obtained from BD Biosciences. F(ab’)2 fragments of goat anti–mouse IgM for stimulation were from Jackson ImmunoResearch Laboratories, Inc.. FITC- conjugated antibody to IFN-γ, PE- conjugated antibodies to IL-4, IL-17A and Foxp3 were from E-bioscience. FITC-conjugated donkey anti-mouse IgG and Alexa Fluor 594-conjugated donkey anti-rabbit IgG secondary antibodies were obtained from Jackson Immunoresearch. Egr2 and Egr3 antibodies were purchased from Covance and SANTA CRUZ, respectively. Antibodies to pErk42/44, myc-tag, flag-tag, pSTAT1, pSTAT3, STAT1, pSTAT5, STAT5 and histone-3 were from Cell signaling Technology; anti-Erk, Rabbit anti-c-Fos, Mouse anti-Jun B, and Mouse anti-BATF were obtained from SANTA CRUZ. For flow cytometry analysis, single cell suspensions were analyzed on a LSRII (BD Immunocytometry Systems) and the data were analyzed using FlowJo (Tree Star). Cell sorting was performed on a FACSaria sorter with DIVA option (BD Immunocytometry Systems).

**Cell isolation and stimulation**

Naïve CD4+ T and B cells were purified by negative selection using MACS systems (Miltenyi Biotec). CD4+CD44<sup>high</sup> and CD4+CD44<sup>low</sup> T cells were sorted by FACS after staining with FITC-conjugated CD4, PE-conjugated CD44 and APC conjugated CD25 antibodies. Cells were gated on CD25<sup>−</sup> cells to exclude activated cells and Treg. Purified CD4+ T cells were stimulated with plate-bound anti-CD3 at 5ug/ml, or at the indicated concentrations, and anti-CD28 (2 ug/ml) antibodies for the indicated times. B cells were activated with soluble anti-F(ab’)2 fragments of goat anti–mouse IgM (anti-IgM F(ab’)2, Jackson ImmunoResearch Laboratories, Inc.) at 5ug/ml, or LPS (Sigma-Aldrich) at 5ug/ml, or at the indicated concentrations, for the indicated times.

**Lentiviral transduction**
The lentiviral constructs for SOCS expression were produced and concentrated as described previously (Miao et al., 2006). In addition to SOCS, the constructs carry an IRES driven GFP which allows us to isolate transduced cells by fluorescence activated cell sorting. Naïve CD4\(^+\) T cells from CD2-Egr2\(^{-/-}\)Egr3\(^{-/-}\) mice at 1 x 10\(^6\) per well in a 24-well plate coated with anti-CD3 and anti-CD28 were infected with concentrated lentivirus at a multiplicity of infection (MOI) of 50–100 (~10\(^5\)–10\(^6\) transducing units/ng of p24) as previously described (Miao et al., 2006). The infected cells were incubated at 37°C for 7 hr with gentle shaking before addition of 1ml medium. The cells were harvested after 24 hours and the GFP positive cells were isolated by cell sorting. The isolated cells were used for RT-PCR analysis and pSTAT staining as described in our previous report.

**Intracellular cytokine analysis.**

Splenocytes were stimulated with PMA (20 ng/ml) plus ionomycin (0.5 µg/ml) in the presence of Brefeldin A for 5 hours. After staining with cell surface markers, intracellular cytokine staining was performed with the Fixation & Permeabilization Kit and IFN-γ, IL-4 and IL-17A antibodies in accordance with the manufacturer’s instructions (E-bioscience).

**Quantitative real-time PCR.**

Total RNA was extracted from stimulated or unstimulated CD4\(^+\) T or B cells, or from CD4\(^+\)CD44\(^{low}\) and CD4\(^+\)CD44\(^{high}\) cells, using the RNeasy Kit from Qiagen and reverse transcribed using oligo(dT) primers (Amersham Biosciences). Quantitative real-time PCR was performed on a Rotor-Gene system (Corbett Robotics) using SYBR green PCR master mix (Qiagen). Primers used in PCR were listed in Table S1.

The data were analyzed using the Rotor-Gene Software. All samples were run in duplicate, and relative mRNA expression levels were obtained by normalizing against the level of β-actin from the same sample under the same program using: relative expression=2\(^{(\text{CTβ-actin-CTS}\text{target})}\)×10,000.
ELISA.
The concentrations of cytokines in the cell culture supernatants and sera were measured using ELISA kits (IL-2, IFN-γ, IL-3, and IL-4 were from R & D; IL-6, IL-10, IL-17A, IL-17F and GM-CSF kits were from BD bioscience) according to the manufacturer’s instructions.

Histological and serological analysis
Tissues were fixed with 10% formalin in PBS and embedded in paraffin. Sections were stained with haematoxylin and eosin. Histological examination of tissue sections was done in a blind manner. Proteinuria was determined using reagent strips for urinalysis, URS-5K (Access Diagnostics Tests, UK). For analysis of Ig deposits 6 μm frozen kidney sections were stained with a Texas Red conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Lab, INC.). Total serum Ig and Ig isotypes were captured with goat anti-mouse Ig and concentrations were determined by ELISA with HRP-conjugated antibodies against Ig, IgG1, IgG2a, IgG2b, and IgM (Sigma). Reactivity of these antibodies was normalized for equivalent optical density (OD) against total Ig and the corresponding isotype controls (Sigma).
The levels of anti-histone and anti-dsDNA antibodies in serum were measured as described in our previous publication (Zhu et al., 2008).

Cellular transfection and co-precipitation
Mouse Batf and Egr2 cDNAs were tagged with Myc and Flag, respectively, and introduced into pcDNA expression vectors by PCR-cloning approaches. The constructs were confirmed by sequencing. The human embryonic kidney 293 (HEK293) cell line was transfected with Batf, Egr2 or both using the Lipofectamine plus reagent (Gibco). The expression of Batf and Egr2 was confirmed by immunohistochemistry and immunoblotting with antibodies to Myc, Flag, Batf and Egr2. Co-precipitation was carried out by precipitating either Batf or Egr2 with anti-Myc or anti-Flag antibodies, respectively. The precipitates were then
immunoblotted with the reciprocal antibodies as indicated. The AP-1 reporter gene, described in our previous report (Anderson et al., 2006), was transfected into naïve CD4+ T cells, isolated from 4 week old mice, using an Amaxa Nucleofector. The transfected cells were stimulated with anti-CD3 and anti-CD28 for 24 hours and then restimulated for 30 minutes before lysis for luciferase assay (Promega) according to the manufacturer’s instructions. The SOCS1 and SOCS3 promoter-reporter constructs were generated by PCR cloning of the SOCS1 (1.5kb from 7786929 to 7785430 of contig NT_039624.7) and SOCS3 (1.3kb from 29372641 to 29371342 of contig NT_165773.2) proximal promoter regions, including the Egr2 binding sites we have identified, into pGL3 basic luciferase reporter vectors (Promega). The activity was measured after transfection into HEK293 cells that were previously transfected with Egr2 or GFP.

Statistics.

Student’s unpaired t-test was used to analyze the statistical significance of differences between groups. Differences with a p-value < 0.05 were considered significant.
Fig. S1. Defective Egr-2 and -3 expression in CD4 and B cells from CD2-Egr2^-/-Egr3^-/- (K2-3) mice, and Egr2 and 3 expression in CD4^+ and B cells from wild type mice after siRNA knockdown. CD4 or B cells from spleens of wild type (WT) and K2-3 mice were stimulated with anti-CD3 and anti-CD28 or anti-IgM for 6 hours. Egr2 and 3 mRNA and protein were measured by RT-PCR (A) and immunoblotting (B), respectively. Egr2 and 3 expression in CD4^+ and B cells from wild type mice transfected with indicated siRNA or control siRNA (Con). Results in (A) and (C) are presented relative to the expression of b-actin. Data are representative of two experiments.
Fig. S2. Development of thymocytes and B cells in CD2-Egr2<sup>−/−</sup>Egr3<sup>−/−</sup> (K2-3) mice. Thymocytes (A) and bone marrow cells (B) from 4 week old mice of the indicated genotypes were stained as shown and analyzed by flow cytometry. C. Absolute numbers of thymocytes (DN CD4<sup>−</sup>CD8<sup>−</sup>; DP CD4<sup>+</sup>CD8<sup>+</sup>) at 4 weeks of age. D. Absolute numbers of bone marrow B cells (pre-pro-B cells NK1.1<sup>+</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>−</sup>; pro-B cells NK1.1<sup>+</sup>B220<sup>−</sup>CD19<sup>−</sup>CD43<sup>+</sup>; pre-B cells NK1.1<sup>+</sup>B220<sup>−</sup>CD43<sup>−</sup>; Immature-B cells NK1.1<sup>+</sup>B220<sup>+</sup>IgM<sup>−</sup>; mature B cells NK1.1<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup>). Data are representative of two independent experiments and N=3 in each experiment.
Fig. S3. A. Absolute numbers of T and B cells in the spleen of wild type and Egr2 and 3 deficient mice at the indicated ages (N = 4 in each group). B. Peripheral CD4+CD25+ Treg cells from the indicated mice were incubated with wild type naïve CD4 T cells at a 1:1 ratio and stimulated with anti-CD3 and anti-CD28 for three days before proliferation and IL-2 production were measured. C. Onset of autoimmune disease as measured by elevated serum IFN-γ and self-reactive antibody that are three times higher than wild type age-matched mice, and survival rate (D) of wild type (WT), Egr3−/− and CD2-Egr2−/−/Egr3−/− (K2-3), Egr2 transgenic (Tg), CD2-Egr2−/− (K2), and Egr3−/− (K3) mice (n = 8). E. p21cip1 expression in CD4 T cells relative to expression of β-actin. ND = not detected. (A), (B), and (E) are representative of two experiments and N = 4 in each group.
Fig. S4. Expression of T and B cell activation markers by lymphocytes (A), production of IL-17, IL-4, and IFN-γ in CD4+ T cells (B), and IL-1b expression in CD4+ T cells (C), from 8 week old mice of the indicated genotypes. The CD2-Egr2−/−/Egr3−/− (K2-3) and WT data are adapted from Fig 1G and Fig 2B. (D) and (E). A mixture of wild type (CD45.1+) and K2-3 (CD45.2+) lymphocytes, from 4 week old mice, were transferred to rag2−/− mice. 2 and 6 weeks after transfer activation marker expression on wild type and K2-3 lymphocytes in rag2−/− mice was analyzed (D). Six-weeks after transfer, WT and K2-3 lymphocytes from recipient mice were isolated by cell sorting and proliferation in response to antigen receptor stimulation was assessed (E). Data are representative of two independent experiments and N = 3 in each experiment.
Fig. S5. Proliferation of B and T cells in vitro. B or T cells from mice of the indicated genotypes were stimulated in vitro with anti-CD3 and anti-CD28, for CD4 cells (A and B), or anti-IgM and LPS, for B cells (B, and C), for three days. Proliferation was measured by BrdU incorporation (A) or incorporation of ³HTdR (B, and C). The data representative of three experiments. D. Apoptosis of activated CD4 T cells. Resting CD4 T cells from the indicated mice were stimulated with anti-CD3 and anti-CD28 for 16 hours and Annexin V positive cells were measured. The data are representative of three experiments.
**Fig. S6.** Identification of potential Egr-2 binding sites in the proximal regions of the SOCS1 (A) and SOCS3 (B) genes. The mouse, rat and human SOCS1 and SOCS3 genes, and surrounding regions, were aligned using Mulan (Ovcharenko, et al. 2005) and examined for the presence of potential Egr-2 binding sites using the multiTF utility (http://multitf.dcode.org). Nucleotide positions are given relative to the transcriptional start site as defined in the UCSC Genome Browser.
Fig. S7. Expression of Batf in B and T cells and transfection of Batf and Egr2 in HEK293 cells. A. Naïve B and T cells from mice of the indicated genotypes were stimulated with anti-CD3 and anti-CD28, for CD4+ T cells, or anti-IgM, for B cells, for 16 hours, or left unstimulated, and Batf expression was analysed by RT-PCR. B. CD44low and CD44high CD4+ cells were isolated from splenocytes of wild type mice. The expression of Egr2, Egr3 and Batf was analyzed by RT-PCR. Results are presented relative the expression of β-actin. Data are representative of two experiments. C. Coexpression of Batf and Egr2 in the nuclei of transfected HEK293 cells. Transfected cells were stained with the indicated antibodies and counterstained with DAPI.
Table S1. List of primers used for RT-PCR

| Primer | Sense | Antisense |
|--------|-------|-----------|
| Egr2   | 5’-CTTCAGCCGAAGTGACCACC-3’ | 5’-GCTCTTCCGTTCTCTGACC-3’ |
| β-actin | 5’-AATCGTGCTGATACATCAAAG-3’ | 5’-ATGCCCAGGATTCCCATACC-3’ |
| IL-2   | 5’-GCATGGTCTGGATTTGACTC-3’ | 5’-CAATTTGGAACCTGATTCC-3’ |
| IL-4   | 5’-CAAACGTCTTCACACAGCAAC-3’ | 5’-CTTGGAACCTCATGATGC-3’ |
| IL-17A | 5’-AGCGTGCTCAAAACACTGAG-3’ | 5’-CTCATGGTTCTGATTCC-3’ |
| IL-17F | 5’-AACCAAGGACATTTTCTGAC-3’ | 5’-TTTCTTGGCATGACACGC-3’ |
| IFN-γ  | 5’-GACACCAAAACACATGAC-3’ | 5’-AGCTGGAATGCTTGGCA-3’ |
| BATF   | sense: 5’-GAGCTGCCTCTGTCTTC-3’ | anti-sense: 5’-CCAGAAGAGCGACAGAGAC-3’ |
| IL-6   | 5’-TGCTGCATTGCAGTACATGAC-3’ | anti-sense: 5’-ACTCTCTGTGACTCCAGAC-3’ |
| IL-1b  | 5’-CTGTGTAATGAAAGACGGC-3’ | 5’-GGTGCTGATGTACCAGTGG-3’ |
| IL-21  | 5’-ATCTATGACTCTCATGATGAC-3’ | 5’-GCATTAGCTGTGGTCTTGGTCC-3’ |
| GM-CSF | 5’-TGCTGCTCAAGTCAGCCCA-3’ | 5’-CCATGGACCCTGCTGAGAATA-3’ |
| SOCS1  | 5’-TGTCGCGCGAGTACGCTGA-3’ | anti-sense: 5’-ACG TAG TGC TCC AGC AGC TC-3’ |
| SOCS2  | 5’-GAC GTG ACC TGG ACC GAC TAA C-3’ | 5’-GGT ACA TTT GTT AAT GGC GAG TCG-3’ |
| SOCS3  | 5’-TTCTTACACACTGACGCTGAGA-3’ | 5’-CTTGAGTACACAGCTCAAGCGGG-3’ |