

Ets-1 Regulates Plasma Cell Differentiation by Interfering with the Activity of the Transcription Factor Blimp-1^{*S}

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Shinu A. John[‡], James L. Clements[§], Lisa M. Russell[‡], and Lee Ann Garrett-Sinha^{‡1}

From the [‡]Department of Biochemistry, State University of New York, Buffalo, New York 14214 and [§]Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York 14263

Development of immunoglobulin-secreting plasma cells from B cells is a tightly regulated process controlled by the action of a number of transcription factors. In particular, the transcription factor Blimp-1 is a key positive regulator of plasmacytic differentiation via its ability to suppress expression of genes involved in the mature B cell program. The transcription factor Ets-1 is a negative regulator of plasmacytic differentiation, as indicated by the development of increased numbers of IgM-secreting plasma cells in Ets-1 knock-out mice. We have previously shown that Ets-1-deficient B cells undergo enhanced differentiation into IgM-secreting plasma cells in response to Toll-like receptor 9 (TLR9) signaling. We now explore the mechanism by which Ets-1 limits differentiation downstream of TLR9. Our results indicate that Ets-1 physically interacts with Blimp-1, which leads to a block in Blimp-1 DNA binding activity and a reduction in the ability of Blimp-1 to repress target genes without interfering with Blimp-1 protein levels. In addition, we show that Ets-1 induces the expression of several target genes that are repressed by Blimp-1, including Pax-5. These results reveal a previously unknown mechanism for the control of Blimp-1 activity by Ets-1 and suggest that expression of Ets-1 must be down-regulated before plasmacytic differentiation can occur.

The generation of plasma cells, the terminally differentiated effector cells of the B cell lineage, is one of the critical events in an immune response. *In vitro*, B cell differentiation into plasma cells can be efficiently induced by stimuli that trigger the activation of certain Toll-like receptors (TLR).² For example, lipopolysaccharide, which triggers TLR4, and unmethylated CpG containing DNA, which triggers TLR9, are effective stimuli to induce plasma cell differentiation *in vitro*. In addition, TLR activation has been shown to play a role in supporting optimal B cell differentiation into immunoglobulin-secreting plasma cells *in vivo* (1, 2), although this result is controversial (3).

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¹ To whom correspondence should be addressed: 140 Farber Hall, 3435 Main St., Buffalo, NY 14214. Fax: 716-829-2725; E-mail: leesinha@buffalo.edu.

² The abbreviations used are: TLR, Toll-like receptor; CpG, cytosine-phosphate-guanine; ODN, oligodeoxynucleotide; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; CMV, cytomegalovirus; HA, hemagglutinin; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; BSAP, B cell-specific activator protein.

A complex transcriptional program that includes both positive and negative regulators dictates B cell differentiation into plasma cells. The transcription factors Blimp-1, Irf-4, and Xbp-1 serve as positive regulators by facilitating plasma cell formation and immunoglobulin secretion (4–6). However, Blimp-1 is considered the master regulator of this process, as ectopic expression of Blimp-1 in mature B cells is sufficient to drive plasmacytic differentiation (4, 7). Moreover, mice harboring a B cell-specific deletion of Blimp-1 exhibit severe defects in plasma cell formation and immunoglobulin secretion (8, 9). Blimp-1 functions as a master regulator by repressing key genes involved in the mature B cell program, in germinal center reactions, and in cell cycle progression (10–14). Together, these changes drive terminal differentiation of B cells into immunoglobulin-secreting plasma cells.

Although induction of Blimp-1 is necessary for plasmacytic differentiation, what is equally important is the suppression or inactivation of Blimp-1 in the mature B cell lineage until the appropriate signals are encountered for terminal differentiation. The suppression/inactivation of Blimp-1 is critical because expression of Blimp-1 in immature B cells can induce apoptosis (10, 15), whereas expression of Blimp-1 in mature B cells can block the germinal center reaction by interfering with Bcl-6 function (14). Moreover, premature Blimp-1 expression could potentially lead to inefficient immune responses or promote secretion of autoantibodies. Hence, mechanisms must be in place to ensure that Blimp-1 activity is properly regulated and occurs only at the appropriate stage of B cell differentiation. The transcription factors Bcl-6 and Bach-2 are known to repress Blimp-1 expression in cells committed to a germinal center fate (16, 17). In addition, the transcription factor Pax-5 can repress Blimp-1 expression in mature B cells, although it is not clear whether this is due to direct binding to the Blimp-1 gene or via up-regulation of Bcl-6 (18, 19). Evidence also suggests that the transcription factor Ets-1 may play a key role in regulating Blimp-1 activity, since mice lacking Ets-1 exhibit a strikingly increased number of IgM-secreting plasma cells in their lymphoid organs (20, 21). Moreover, B cells lacking Ets-1 undergo enhanced differentiation into IgM-secreting plasma cells when cultured in the presence of a synthetic TLR9 ligand (CpG oligodeoxynucleotide (ODN) (22).

The mechanism by which Ets-1 regulates the development of IgM-secreting plasma cells is unknown but likely involves modulating the expression or activity of genes that govern the plasma cell fate. Interestingly, several Ets proteins can direct lineage-commitment decisions via their ability to form protein-protein complexes with key lineage-specific transcription fac-

tors, leading to inactivation of the function of those transcription factors. For instance, the Ets protein PU.1 physically interacts with the erythroid-specific transcription factor GATA-1 to block its activity and prevent erythrocyte terminal differentiation (23–26). Likewise, another Ets protein, Fli-1, interacts with the erythroid-specific transcription factor EKLf to regulate its function and limit terminal differentiation of erythroid cells (27). Furthermore, the Ets factor MEF (myeloid Elf-1-like factor) interacts with the Runx-2 transcription factor to inhibit its activity and regulate bone development (28).

We investigated whether Ets-1 might restrain commitment to B cell terminal differentiation in a similar manner by binding to and inactivating the key transcription factor regulating this developmental decision Blimp-1. Here, we present evidence that Ets-1 has a B cell-intrinsic role in regulating plasmacytic differentiation downstream of TLR9 and that this is mediated at least in part by a functional antagonism between Ets-1 and Blimp-1. This antagonism is brought about by direct binding of Ets-1 to Blimp-1, which inhibits the ability of Blimp-1 to bind to its target sites in DNA. The mechanism of Ets-1 action is functionally distinct from the mechanisms employed by other negative regulators of Blimp-1 activity (Bcl-6, Pax5, and Bach-2). In addition to its ability to directly bind to Blimp-1 and interfere with its function, we provide evidence that Ets-1 also up-regulates the expression of several target genes, which are known to be repressed by Blimp-1. Among the genes up-regulated by Ets-1 is Pax5, which encodes a transcription factor required to specify the mature B cell fate. Together, our data indicate that Ets-1 employs two separate and complementary modes of action to control B cell differentiation into plasma cells.

EXPERIMENTAL PROCEDURES

Plasmids—The MIGR1 retroviral vector has been previously described (29). Plasmids derived from the MIGR1 vector generate bicistronic messages encoding a cDNA of interest as well as a marker gene GFP. Full-length or deleted versions of the mouse Ets-1 cDNA were subcloned into MIGR1. MIGR1 and its derivatives were co-transfected into a viral packaging cell line along with the plasmid pCL-Eco (a gift of Dr. Rodney DeKoter, University of Cincinnati), which harbors the viral *gag*, *pol*, and *env* genes under the control of the CMV promoter (30).

To generate a Blimp-1 construct for *in vitro* transcription/translation, an HA-tagged version of Blimp-1 was cloned into the pCITE-4a vector (Novagen, Madison, WI). Plasmids encoding glutathione *S*-transferase (GST) fusions of Ets-1, Ets-2, or PU.1 were generated by PCR amplification of the respective cDNAs (or portions thereof) using appropriate primers followed by cloning into pGEX vectors (GE Healthcare). All PCR products were verified by DNA sequencing.

The plasmid pCMV-HA-Ets-1, harboring the full-length murine Ets-1 cDNA fused in-frame to an HA tag at the N terminus, has been previously described (22). A deleted version of Ets-1 lacking the Ets domain (amino acids 331–415) was amplified from a GST vector and subcloned into pCMV-HA to generate pCMV-HA Ets1 Δ 331–415. The Blimp-1 expression plasmid (pcDNA3.1 Blimp-1) containing a full-length murine Blimp-1 cDNA fused in-frame to a FLAG tag at the C terminus was a gift of Dr. Kathryn Calame (Columbia University, New

York). We generated a FLAG-tagged version of Blimp-1 lacking the PEST sequences (Blimp1 Δ 350–557) using a PCR mutagenesis protocol. The integrity of the PCR product was verified by sequencing. The plasmid CIITA-luc (containing –545 to +123 bp of promoter III of the human *MHC2TA* gene (31)) was a gift of Dr. Jenny Ting (University of North Carolina, Chapel Hill, NC). The plasmid BSAP-Luc (containing –1771 to +50 bp of the murine Pax-5 promoter (32)) was a gift of Dr. Kathryn Calame. The plasmid cMYC-Luc (containing –992 to +148 bp of the murine c-Myc promoter) was generated by PCR amplification of mouse genomic DNA followed by cloning into the pGL3Basic vector. The pCMV- β gal plasmid was used as an internal control for transfection efficiency.

Cell Lines—The mature B cell line A20 and the plasmacytoma cell line P3X were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin/streptomycin, glutamine, and 50 μ M β -mercaptoethanol. The African green monkey kidney cell line Cos-1 and the retroviral packaging cell line Platinum-E were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and glutamine. Platinum-E cells are a derivative of the 293T human embryonic kidney cell line with stably integrated copies of the viral structural genes (*gag*, *pol*, and *env*) driven by the potent EF1 α promoter (33). Platinum-E cells were a kind gift of Dr. Toshio Kitamura, University of Tokyo, Tokyo, Japan.

B Cell Purification and Stimulation—B cells were purified from the spleens of C57BL/6 mice by negative selection against CD43 using a VarioMACS magnetic column and CD43 microbeads (Miltenyi Biotec, Auburn, CA). For *in vitro* differentiation, purified splenic B cells were stimulated with 5–10 μ g/ml synthetic TLR9 ligand, CpG ODN 1826 (5'-TCCA-GACGTTCTGACGTT-3') (Coley Pharmaceuticals, Wellesley, MA), a phosphorothioate containing oligodeoxynucleotide with unmethylated CpG sequences.

Western Blot Analysis—Whole cell lysates were prepared at the indicated time points by direct boiling in Laemmli sample buffer or by repeated freeze-thaw cycles in lysis buffer (20 mM Tris, pH 7.5, 100 mM KCl, 20% glycerol, 0.1% Nonidet P-40, 2 mM dithiothreitol, and a complete protease inhibitor mixture). The samples were subjected to Western blotting, and membranes were incubated with rabbit polyclonal anti-mouse Ets-1 (either the C-20 or N-276 anti-Ets-1 antibody, Santa Cruz Biotechnology, Santa Cruz, CA), mouse or rat monoclonal anti-Blimp-1 (clone 3H2-E8, Novus Biologicals, Littleton, CO or clone 6D3, Santa Cruz Biotechnology), mouse monoclonal anti-Pax-5 (clone A-11, Santa Cruz Biotechnology), or mouse monoclonal anti- β -tubulin (clone KMX-1; Chemicon International, Temecula, CA).

Immunofluorescent Staining—Splenic B cells isolated from C57BL/6 wild-type mice were stimulated for 48 h with CpG ODN and harvested onto polylysine-coated slides. Cells were fixed with 4% paraformaldehyde and stained with a rabbit polyclonal antibody specific for Ets-1 (N-276, Santa Cruz Biotechnology) and a rat monoclonal antibody specific for Blimp-1 (clone 6D3, Santa Cruz Biotechnology). An anti-rabbit secondary antibody coupled to Alexa-Fluor 568 and an anti-rat secondary antibody coupled to Alexa-Fluor 488 were used to

detect Ets-1 and Blimp-1. Images were captured on a Nikon Microphot FXA microscope and analyzed with SPOT software (Diagnostic Instruments, Sterling Heights, MI).

Purification of GST Fusion Proteins and GST Pulldown Assay—GST fusion proteins were expressed in *Escherichia coli* BL21 cells and purified using a standard protocol. Radiolabeled Blimp-1 protein was generated using an *in vitro* transcription and translation reaction. For the GST pulldown assays, equal amounts of GST fusion proteins were incubated with *in vitro* transcribed and translated ³⁵S-labeled Blimp-1 in a buffer (50 mM Tris, pH 8.0, and 0.2 mM ZnCl₂) followed by incubation with glutathione-Sepharose beads. After washing, the bound proteins were eluted in SDS sample loading buffer, resolved on SDS-PAGE gels, and visualized by autoradiography.

Co-immunoprecipitation Assays—Cos-1 cells were transfected using FuGENE 6 (Roche Applied Sciences) with 250 ng to 1 μg of each plasmid (pCMV-HA-Ets1, pCMV-HA-Ets1 Δ331–415, or pcDNA3.1 Blimp1 Δ350–557). Forty-eight hours later cells were lysed in a buffer (1% Nonidet P-40, 20 mM Tris HCl, pH 7.5, 300 mM NaCl, 0.1 M Na₂P₂O₇, 0.8 mM EDTA, and a complete protease inhibitor mixture (Roche Applied Sciences)). Supernatants were precleared by incubation with protein-G-agarose beads (Roche Applied Sciences) and then incubated with either a rat monoclonal anti-HA antibody (clone 3F10, Roche Applied Biosciences), a control rat IgG1 antibody (BD Biosciences Pharmingen), a mouse monoclonal anti-FLAG antibody (clone M2, Sigma-Aldrich), or a control mouse IgG1 antibody (BD Biosciences Pharmingen). Immunoprecipitates were Western-blotted using anti-FLAG or anti-HA antibodies.

For co-immunoprecipitation of endogenous Ets-1 and Blimp-1, B cells were purified from the spleens of C57BL/6 mice and cultured *in vitro* with 10 μg/ml CpG ODN to induce up-regulation of Blimp-1. After 48 h of culture the cells were resuspended in lysis buffer, and lysates were precleared by incubation with protein G-Sepharose. Ets-1 was immunoprecipitated using a rabbit polyclonal anti-Ets-1 antibody (N-276, Santa Cruz Biotechnology) or a control rabbit antibody. Immunoprecipitates were separated on SDS-PAGE gels and Western-blotted with a rat monoclonal anti-Blimp-1 antibody (clone 6D3, Santa Cruz Biotechnology).

Electrophoretic Mobility Shift Assays (EMSAs)—Whole cell extracts from P3X plasmacytoma cells, and A20 mature B cells were isolated by repeated freeze-thaw cycles in lysis buffer (as described above for Western blotting). For EMSAs, a double-stranded oligonucleotide containing Blimp-1 binding site from the c-Myc promoter (5'-GCGTGAAAGGGAAAGGAC-TAGCGC-3') or from the Pax-5 promoter (5'-TCGAGTTT-GGAAAGTGAATCGCTC-3') was labeled with [α-³²P]dCTP and used as probe. Binding reactions were carried out in DNA binding buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0, 4% Ficoll, 1 mM dithiothreitol, 75 mM KCl) using cell extracts containing ~10 μg of protein. For competition, P3X extracts were incubated with a 100-fold excess of wild-type or mutant c-Myc oligonucleotides (mutant oligonucleotide 5'-GCGTGAC-AGGGGCAGGACTAGCGC-3'; mutant bases indicated are in bold type). For supershift assays, P3X extracts were preincubated with 1 μl of Blimp-1 antibody (clone 3H2-E8, Novus Biologicals). Where indicated, equivalent amounts of GST fusion

proteins were incubated with P3X extracts for 30 min on ice before the addition of labeled probe.

Transfection and Reporter Assays—A20 cells were transfected by electroporation as described previously (34). Transfections included 5 μg of luciferase reporter construct, 0.25 μg of an internal control (pCMV-βgal), 1 or 4 μg of the Blimp-1 expression vector (pCDNA3.1 Blimp-1), and varying concentrations of the Ets-1 expression vector (pCMV-HA-Ets1). Luciferase and β-galactosidase activities were measured 48 h post-transfection using the Luciferase Reporter Assay System (Promega) and the Galacto-Light Plus β-Galactosidase Reporter Gene Assay System (Applied Biosystems, Foster City, CA). Values are reported as ratios of luciferase to β-galactosidase.

Retroviral Production and Transduction—For production of retrovirus, the Platinum-E packaging cell line was transfected with various retroviral expression plasmids along with the pCL-Eco plasmid using FuGENE 6 (Roche Applied Biosciences). Retroviral supernatants were used to infect 3 × 10⁶ B cells, purified from the spleen of C57BL/6 mice, and stimulated with 5 μg/ml CpG ODN. Two days after infection, the GFP-positive population was sorted out using FACsAria Cell Sorter (BD Biosciences Immunocytometry Systems). For some experiments GFP^{lo} and GFP^{hi} populations were independently sorted. Proliferation of sorted GFP⁺ virally infected cells was measured by [³H]thymidine incorporation.

Flow Cytometry—Retrovirus-transduced cells were stained with phycoerythrin-conjugated anti-mouse Syndecan-1 (CD138) (BD Biosciences Pharmingen). Samples were analyzed on a BD Biosciences Immunocytometry Systems FACSCalibur flow cytometer, and the resulting data were evaluated using FlowJo software (TreeStar Inc, Ashland, OR).

ELISA—Equivalent numbers of sorted GFP-positive cells from the retrovirally transduced populations were resuspended in media containing 10 μg/ml CpG ODN. After 24 or 48 h, supernatants were harvested, and ELISA was carried out. Purified mouse IgM (clone 11E10, Southern Biotech) was used for generating a standard curve.

RESULTS

Ets-1 Represses B Cell Terminal Differentiation in a B Cell-intrinsic Fashion—Mice deficient in the Ets-1 protein exhibit an increased number of IgM-secreting plasma cells (20, 21). We previously showed that splenic B cells isolated from Ets-1 knock-out mice (designated *Ets-1*^{p/p} mice) undergo enhanced differentiation into IgM-secreting cells when they are cultured in the presence of a synthetic TLR9 ligand (CpG ODN) (22), suggesting a possible B cell-intrinsic role for Ets-1 in regulating differentiation. To further explore the B cell-intrinsic requirement for Ets-1 in regulating plasma cell development in response to TLR9, we have examined the effect of enforced expression of Ets-1 in cultures of wild-type B cells induced to undergo differentiation by the addition of CpG ODN.

To drive high level, constitutive expression of Ets-1, we generated a retroviral construct encoding the full-length murine Ets-1 cDNA followed by an internal ribosomal entry site and GFP (*MIGR1 Ets1*, Fig. 1A). *MIGR1-Ets1* virus or the empty *MIGR1* virus were used to infect purified, primary splenic B

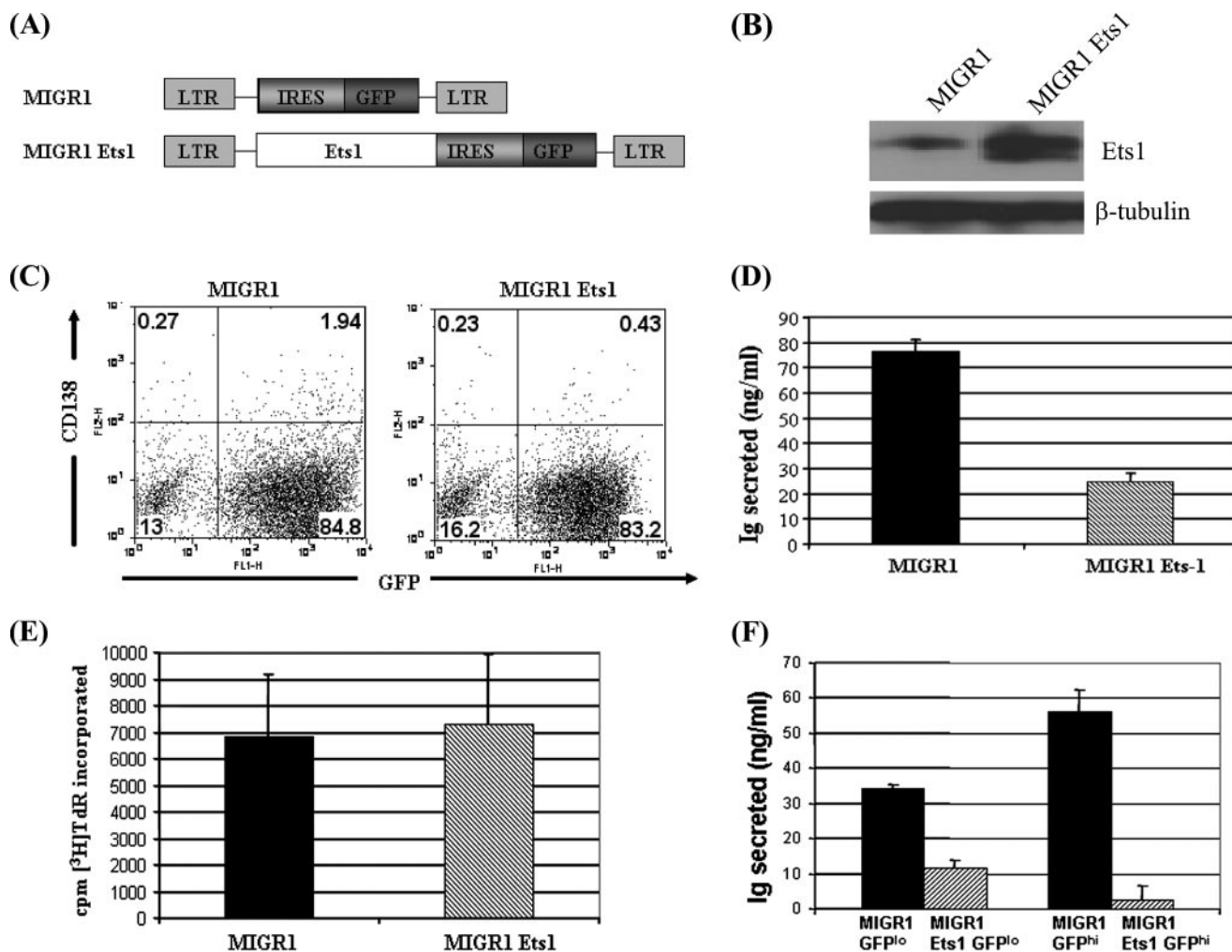


FIGURE 1. Enforced expression of Ets-1 in primary B cells inhibits plasmacytic differentiation. A, retroviral constructs used for transduction experiments. LTR, long terminal repeat; IRES, internal ribosomal entry site. B, Western blot analysis of Ets-1 levels in retrovirally infected B cells. C, flow cytometry analysis of retrovirally infected primary B cells. Splenic B cells were isolated from C57BL/6 mice, stimulated with CpG ODN, infected with the retroviral constructs, and returned to culture in the presence of CpG ODN. Two days later cells were stained with anti-CD138 antibody and analyzed by flow cytometry to quantitate plasma cell differentiation. D, to assess IgM secretion, GFP-positive cells were sorted from each population 2 days post-retroviral infection and returned to culture in the presence of CpG ODN. ELISA was performed after an additional 2 days. Results show the averages and S.E. from three independent experiments. E, proliferation of CpG ODN-stimulated, GFP-positive, sorted, virally infected B cells. F, GFP^{lo} and GFP^{hi} populations of virally infected, CpG ODN-stimulated B cells were sorted as described in C above. IgM secretion was measured by ELISA 24 h after sorting. Representative results from one of two independent experiments are shown.

cells stimulated overnight with CpG ODN to induce cell cycle progression. As expected, cells harboring MIGR1-Ets1 exhibited substantially higher expression of Ets-1 than cells harboring the control virus (Fig. 1B). After infection with retrovirus, B cells were returned to culture with CpG ODN, which induces differentiation into IgM-secreting plasma cells. Forty-eight hours later the status of plasmacytic differentiation in each population was assessed by staining for CD138 (Syndecan-1), a marker expressed at high levels on plasma cells. Cells infected with MIGR1-Ets1 gave rise to 4–5-fold fewer GFP⁺CD138^{hi} plasma cells than did cells infected with the control virus (Fig. 1C), indicating reduced levels of terminal differentiation. Similar results were obtained with B cells stimulated with lipopolysaccharide to induce plasmacytic differentiation (supplemental Fig. S1).

To further characterize differentiation of these cells, we sorted GFP-positive cells from each population at 48 h after retroviral transduction, cultured them in the presence of CpG

ODN, and measured IgM secretion after an additional 48 h (Fig. 1D). Enforced expression of high levels of Ets-1 strongly suppressed IgM secretion, indicating a specific block to plasmacytic differentiation. The alterations in CD138^{hi} cell numbers and IgM secretion could not be explained by differential proliferation, as the two populations of cells showed very similar levels of [³H]thymidine incorporation (Fig. 1E). In addition, forward and side scatter profiles of flow-sorted virally infected cells demonstrated similar percentages of dead cells, suggesting that Ets-1 did not have a major influence on the rate of B cell survival either (data not shown).

To determine whether lower levels of Ets-1 expression (which may be more physiologically relevant) could inhibit differentiation to IgM-secreting plasma cells, we sorted out GFP^{lo} and GFP^{hi} populations from retrovirally infected cells. Because Ets-1 and GFP are expressed from a bicistronic mRNA, it was expected that the level of Ets-1 expression would be correlated with the level of GFP expression. This was confirmed by West-

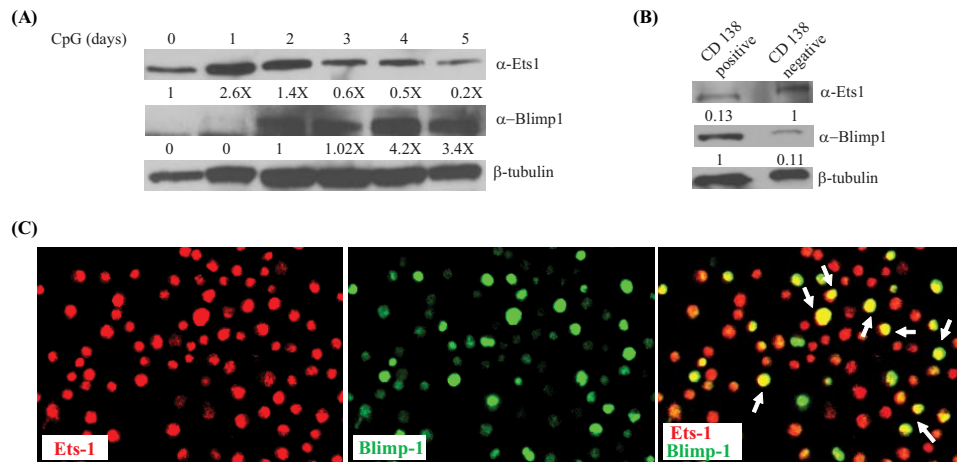


FIGURE 2. Ets-1 and Blimp-1 exhibit reciprocal expression patterns during plasmacytic differentiation. A, Western blot analysis of Ets-1, Blimp-1, and β -tubulin in whole cell lysates of purified splenic B cells either unstimulated or stimulated with CpG ODN (10 μ g/ml) for the indicated time points. Levels of Ets-1 were arbitrarily assigned a value of 1 in unstimulated B cells (day 0), whereas levels of Blimp-1 were arbitrarily assigned a value of 1 in B cells stimulated for 2 days when Blimp-1 levels were first detected at high levels. -Fold changes in Ets-1 and Blimp-1 expression (normalized for β -tubulin) are shown below each lane. B, Western blot analysis of Ets-1, Blimp-1, and β -tubulin in whole cell lysates of flow-sorted CD138⁻ B cell blasts and CD138⁺ plasmablasts isolated from cultures of CpG ODN-stimulated splenic B cells. Levels of Ets-1 were arbitrarily assigned a value of 1 in sorted B cell blasts, whereas levels of Blimp-1 were arbitrarily assigned a value of 1 in sorted plasmablasts. -Fold changes in Ets-1 and Blimp-1 expression (normalized for β -tubulin levels) are shown below each lane. C, immunofluorescent staining of Ets-1 (red) and Blimp-1 (green) in B cells stimulated for 48 h with CpG ODN. Arrows indicate cells that co-express both proteins (yellow). Magnification, 200 \times .

ern blotting for Ets-1 in sorted GFP^{hi} and GFP^{lo} populations (data not shown). IgM secretion from cultures of GFP^{hi}- and GFP^{lo}-sorted B cells was measured (Fig. 1F), which indicated that Ets-1 repressed IgM secretion in a dose-dependent fashion. Thus, even a low level of Ets-1 is sufficient to partially suppress plasmacytic differentiation, whereas high levels of Ets-1 can almost completely suppress such differentiation. Together, these experiments demonstrate that Ets-1 plays a cell-intrinsic role in inhibiting B cell terminal differentiation in response to a synthetic TLR9 ligand.

Ets-1 and Blimp-1 Exhibit an Inverse Expression Pattern during B Cell Differentiation—Our experiments support the notion that Ets-1 acts as a negative regulator of plasmacytic differentiation in a B cell-intrinsic fashion. This observation suggests that there may be a requirement for down-regulation of Ets-1 as B cells commit to a plasma cell program. If so, then Ets-1 levels should decrease as B cells progress through differentiation. To test this hypothesis, we examined the expression pattern of Ets-1 in primary B cells stimulated with CpG ODN to induce plasma cell development. Ets-1 expression was high in freshly isolated splenic B cells (day 0 in Fig. 2A) and remained high until day 2 of culture, after which expression declined significantly. In contrast, Blimp-1 was undetectable in unstimulated, purified splenic B cells but was up-regulated in response to CpG ODN beginning at day 2.

The Western blot results described above were obtained with mixed populations of cells representing mainly activated B cells (B cell blasts) with a minority of cells that had progressed to plasmablasts and fully differentiated plasma cells. Although we detected a reciprocal expression pattern for Ets-1 and Blimp-1 in these mixed populations, it was not clear that this expression pattern reflected a reduction of Ets-1 in cells that had committed to terminal differentiation. To confirm that Ets-1 was

down-regulated during differentiation, we purified B220⁺CD138⁻ B cell blasts and B220⁺CD138⁺ plasmablasts from CpG-stimulated B cell cultures and assessed the expression of Ets-1 and Blimp-1 by Western blotting. As shown in Fig. 2B, Ets-1 and Blimp-1 are co-expressed in both CD138⁻ and CD138⁺ cells. However, Ets-1 levels are \sim 8-fold higher in CD138⁻ B cell blasts than in CD138⁺ plasmablasts. Blimp-1 expression exhibits an opposite pattern being \sim 10-fold higher in plasmablasts than in B cell blasts.

Finally, to confirm that Ets-1 and Blimp-1 are co-expressed in the same cells, we performed immunofluorescent staining of Ets-1 and Blimp-1 in CpG-stimulated B cells (Fig. 2C). Both Ets-1 and Blimp-1 were present in the nuclei of B cells, with a percentage of cells expressing either Ets-1 or Blimp-1 alone,

whereas others co-expressed these two proteins. Thus, many cells in the CpG-stimulated cultures express both Ets-1 and Blimp-1 at a detectable level. Together these data indicate that Ets-1 levels decrease and Blimp-1 levels increase as B cells commit to terminal differentiation, supporting the notion that down-regulation of Ets-1 may be essential to allow plasma cell development.

Ets-1 Physically Interacts with Blimp-1 in Vitro—The data presented in Fig. 1 indicate that Ets-1 blocked full plasmacytic differentiation and immunoglobulin secretion. Several potential models can be envisioned to account for the ability of Ets-1 to block plasmacytic differentiation. In this report we have focused on determining whether Ets-1 can interfere with the expression or activity of the master regulator of plasma cell development Blimp-1. Our data suggest that Ets-1 does not repress expression of the Blimp-1 gene (see Fig. 6D and data not shown). However, based on the previously known functional antagonism between certain Ets family proteins and lineage-commitment transcription factors (23–28), we hypothesized that Ets-1 might physically interact with Blimp-1 to interfere with its activity.

To test this hypothesis we performed GST pulldown assays to assess protein-protein interaction. Purified GST or GST-Ets1 was incubated with ³⁵S-labeled *in vitro* transcribed and translated Blimp-1 followed by purification on glutathione-Sepharose beads. Radiolabeled Blimp-1 was retained on beads containing bound GST-Ets1 but not on beads containing GST nor on beads alone (Fig. 3A). In contrast, GST-Ets1 did not interact with a radiolabeled control protein (β -galactosidase). Thus, there is a specific interaction of Blimp-1 and Ets-1 in this assay.

We next asked what domains of the Ets-1 protein mediate this interaction. Ets-1 contains several previously defined func-

Ets-1 Blocks the Function of Blimp-1

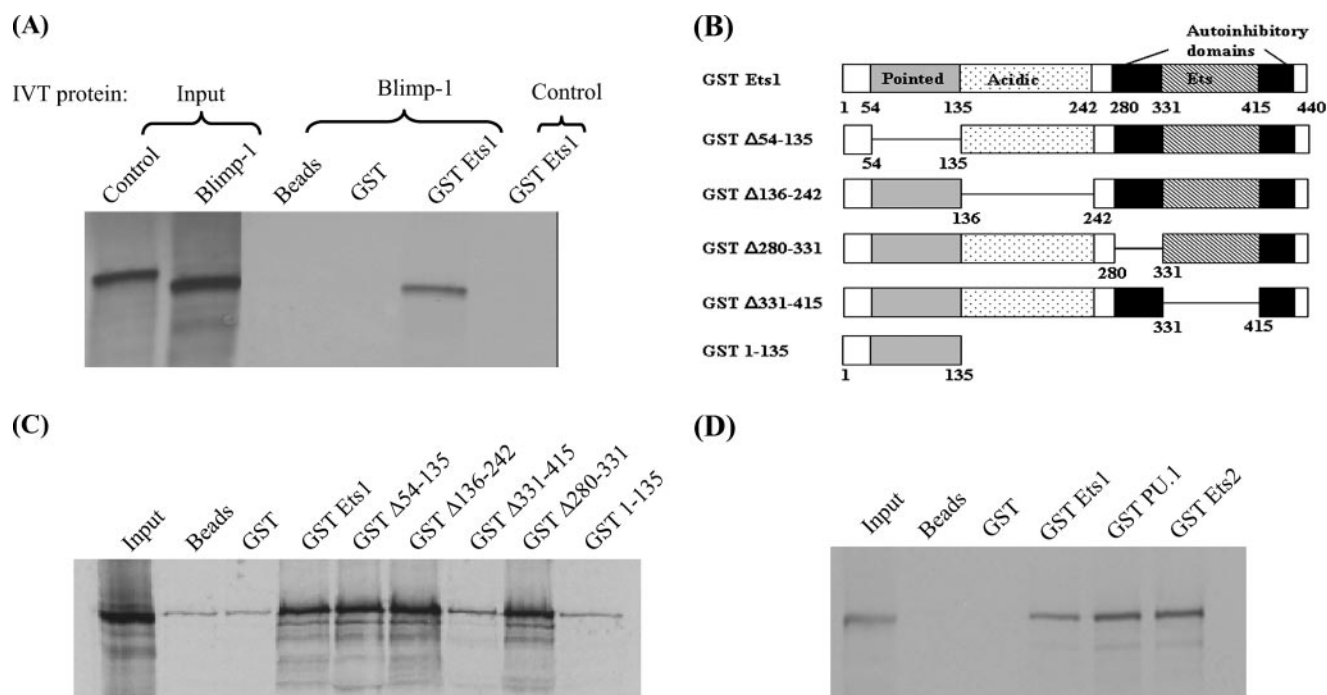


FIGURE 3. Ets-1 physically interacts with Blimp-1 via the Ets domain. A, GST pull-down assays were performed by incubating *in vitro* transcribed and translated (IVT) ^{35}S -labeled wild-type Blimp-1 with glutathione-Sepharose alone (Beads) or with purified GST or GST-Ets1. As a control, *in vitro* transcribed and translated ^{35}S -labeled *E. coli* β -galactosidase (Control) was also incubated with GST-Ets1. B, GST-Ets1 fusion constructs used in the GST pull-down assays. C, GST pull-down assays were performed as in A using GST-Ets1 and various deletion mutants. D, GST pull-down assays were performed as in A using GST-Ets1, GST-Ets2, and GST-PU.1.

tional domains including a Pointed (SAM) domain involved in protein-protein interactions, an acidic transactivation domain, an Ets DNA binding domain, and two autoinhibitory domains that flank the Ets domain (Fig. 3B). To map the domains of Ets-1 required for interaction with Blimp-1, various deletions of Ets-1 were generated as GST-tagged fusion proteins, and GST pull-down assays were performed using similar amounts (supplemental Fig. S2) of each GST protein. A robust interaction was detected with full-length GST-Ets1 as well as GST-Ets1 fusion proteins lacking the Pointed domain (GST Δ 54–135), the transactivation domain (GST Δ 136–242), and the N-terminal autoinhibitory domain (GST Δ 280–331) (Fig. 3C). In contrast, a GST-Ets1 fusion protein lacking the Ets domain (GST Δ 331–415) exhibited only background binding to radiolabeled Blimp-1, similar to that observed with glutathione-Sepharose beads alone. Moreover, a truncation mutant of Ets-1 (GST 1–135), containing only the first 135 amino acids of Ets-1, also failed to interact with Blimp-1. Together, these studies indicate that there is a direct physical interaction between Ets-1 and Blimp-1 that requires amino acid sequences in the DNA binding domain (Ets domain).

Because the Ets domain of Ets-1 is the most highly conserved region of the protein and shares substantial homology with other Ets family members, we performed GST pull-down assays with GST fusions of Ets-2 (a highly related Ets factor, 94% amino acid identity in the Ets domain) and PU.1 (a distantly related Ets factor, 36% amino acid identity in the Ets domain). Similar amounts of GST-Ets1, GST-Ets2, and GST-PU.1 were used in GST pull-down assays (supplemental Fig. S2). As shown in Fig. 3D, radiolabeled Blimp-1 interacted equivalently with all three Ets proteins, strongly supporting the functional signifi-

cance of conserved amino acid residues in the DNA binding domain in mediating the interaction with Blimp-1. Thus, multiple members of the Ets family specifically interact *in vitro* with Blimp-1 via the conserved Ets DNA binding domain. However, as discussed below, only Ets-1 is capable of inhibiting Blimp-1 DNA binding activity when in a complex. This is consistent with Ets-1 having a specific function not found in other Ets family members that allows it to block Blimp-1 activity.

Ets-1 Interacts with Blimp-1 *in Vivo*—To confirm that similar interactions between Ets-1 and Blimp-1 occur *in vivo*, we performed co-immunoprecipitation assays. For this purpose we transfected Cos-1 cells with an HA-tagged Ets-1 or an HA-tagged version of Ets-1 lacking the Ets domain (Ets1 Δ 331–415) and with FLAG-tagged Blimp-1 (Fig. 4A). For our assays we chose to use a deleted form of Blimp-1 lacking the PEST sequences (Blimp1 Δ 350–557) because we found Blimp1 Δ 350–557 to be more stable in cell lysates than was full-length Blimp-1. Lysates from transfected cells were immunoprecipitated with an anti-HA antibody or a control rat IgG antibody. When full-length Ets-1 and Blimp-1 were co-expressed, an antibody specific for the HA tag on Ets-1 was able to efficiently co-immunoprecipitate Blimp-1 (lane 2, Fig. 4B). In contrast, Blimp-1 was not immunoprecipitated by a control rat IgG antibody (lane 1, Fig. 4B). Consistent with our results in GST pull-down assays, a deleted version of Ets-1 lacking the Ets domain (Δ 331–415) failed to bring down Blimp-1 (lane 4, Fig. 4B). Importantly, reverse immunoprecipitations using anti-FLAG antibody showed a specific pull-down of Ets-1, whereas Ets-1 was undetectable when immunoprecipitated using the control mouse IgG antibody (Fig. 4B).

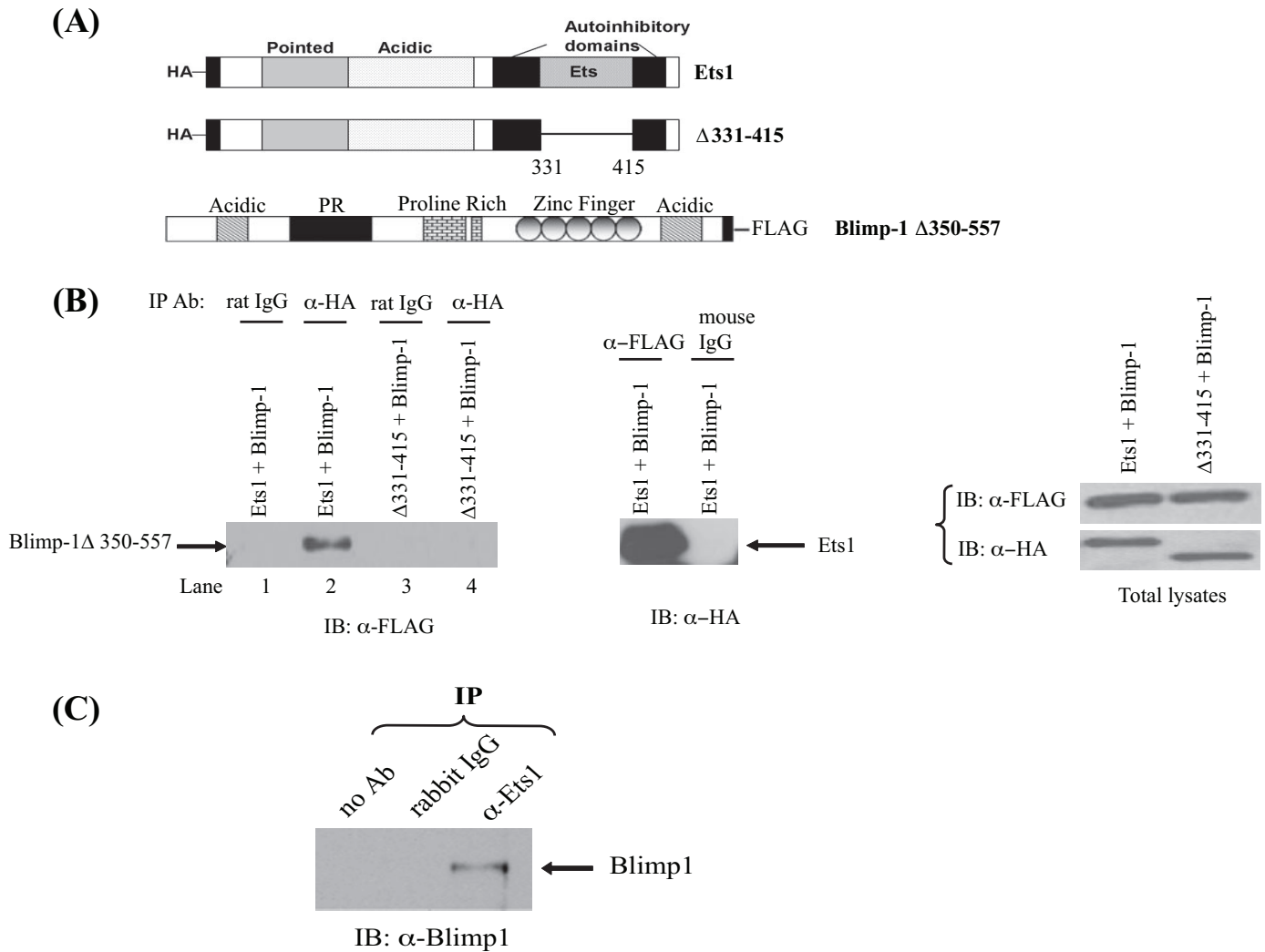


FIGURE 4. Ets-1 interacts with Blimp-1 *in vivo*. *A*, structure of Ets-1 and Blimp-1 constructs used in immunoprecipitation experiments. *B*, co-immunoprecipitation (IP) of Ets-1 and Blimp-1 from transfected Cos-1 cells. HA-tagged Ets-1 or HA-tagged Ets-1 lacking the Ets domain (Ets1 Δ 331–415) together with FLAG-tagged Blimp-1 lacking the PEST sequence (Blimp-1 Δ 350–557) were transfected into Cos-1 cells. 48 h after transfection immunoprecipitation reactions were carried out using either anti-FLAG or anti-HA antibodies (Ab) followed by Western blotting (IB) with the alternate antibody. Levels of transfected Ets-1 and Blimp-1 Δ 350–557 in whole cell lysates of the cells are shown at the side. *C*, co-immunoprecipitation of Ets-1 and Blimp-1 from CpG ODN stimulated primary splenic B cells. Cell lysates were immunoprecipitated with an antibody specific to the N terminus of Ets-1 or with a control antibody. Immunoprecipitated proteins were Western-blotted using an antibody specific for Blimp-1.

To confirm the co-immunoprecipitation of Ets-1 and Blimp-1 in a more physiologically relevant setting, we stimulated splenic B cells with CpG ODN for 48 h and immunoprecipitated Ets-1 from cell lysates followed by immunoblotting for Blimp-1. As shown in Fig. 4C, Ets-1 specifically immunoprecipitated Blimp-1 from CpG-activated B cell lysates. Together, the co-immunoprecipitation of both transiently transfected as well as endogenous versions of Ets-1 and Blimp-1 support the existence of an *in vivo* association between these two proteins, confirming the results we obtained in GST pulldown assays using recombinant proteins.

Ets-1 Inhibits Blimp-1 DNA Binding—We next wished to determine whether the physical interaction of Ets-1 with Blimp-1 would have an effect on the ability of Blimp-1 to bind to its target sequences in DNA. For this purpose we employed EMSA using recombinant Ets-1 and extracts from the plasmacytoma line P3X, which contain abundant Blimp-1. Oligonucleotide probes bearing Blimp-1 binding sites from the c-myc

promoter (Fig. 5A) and the Pax-5 promoter (Fig. 5B) were chosen for this analysis. A specific Blimp-1-DNA complex was seen with each oligonucleotide probe using P3X extracts, which was absent from A20 mature B cell extracts. The complex was supershifted by a Blimp-1 antibody and competed by excess wild-type unlabeled probe but not the mutant unlabeled probe, demonstrating specificity (Fig. 5A and data not shown).

To determine whether Ets-1 could affect Blimp-1 DNA binding, we incubated GST-Ets1 (or as controls GST alone or GST-Ets1 Δ 331–415 lacking the Ets DNA binding domain) with P3X extracts before EMSA analysis. Increasing amounts of GST-Ets1 led to a reduction in the Blimp-1-DNA complex, whereas increasing amounts of GST or GST Δ 331–415 had no effect on the complex (Fig. 5, A and B). The inability of the GST Δ 331–415 protein (lacking the Ets domain) to block Blimp-1 DNA binding further confirms the results obtained in the GST pulldown assays and reiterates the functional importance of the Ets domain in mediating an effective interaction with Blimp-1. The

Ets-1 Blocks the Function of Blimp-1

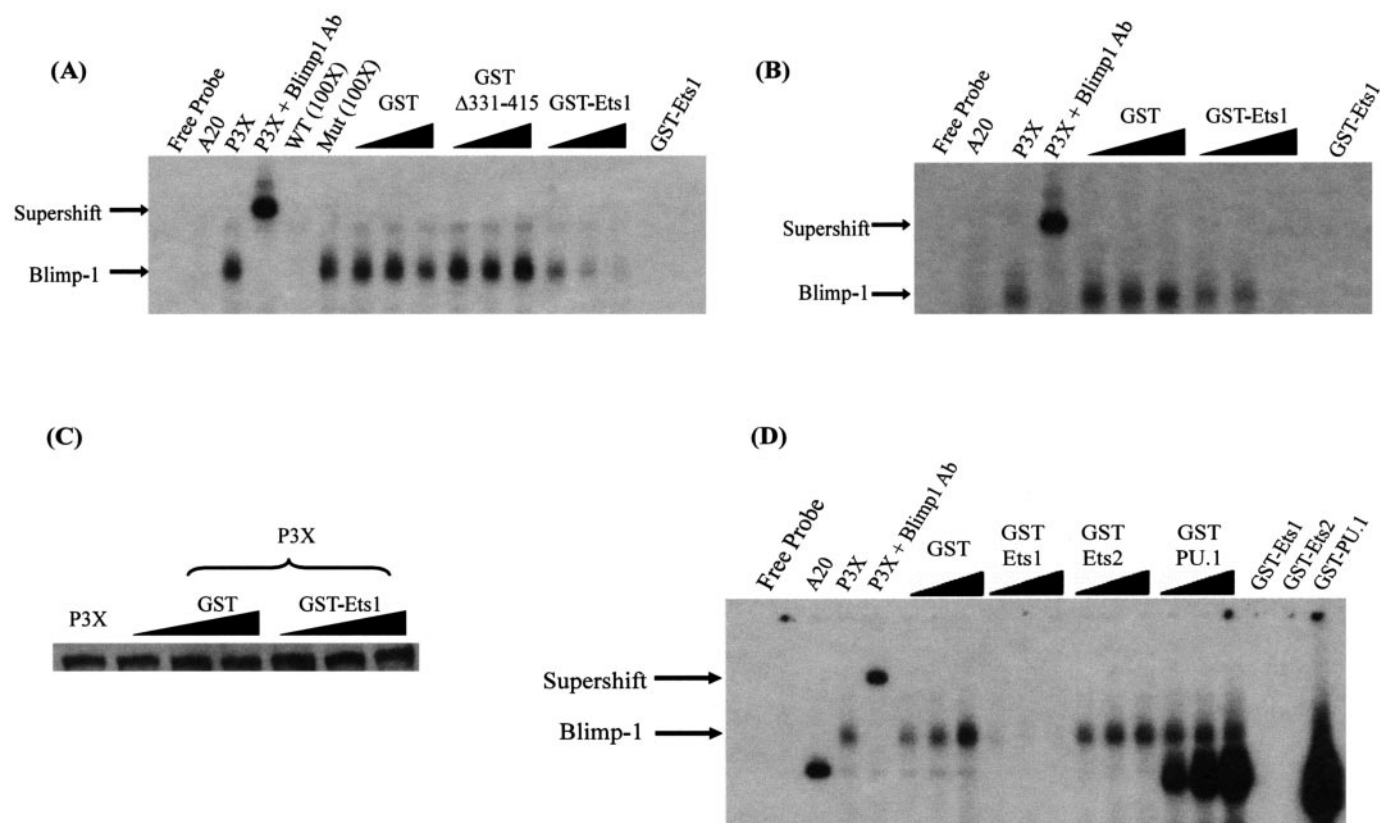


FIGURE 5. Ets-1 interferes with Blimp-1 DNA binding. EMSA was performed using a double-stranded oligonucleotide containing Blimp-1 binding site from the c-Myc promoter (A) or the Pax-5 promoter (B). A specific complex (Blimp-1) was observed when P3X (a plasmacytoma) extracts were used but not with A20 (mature B cell lymphoma) extracts. The complex was supershifted with a Blimp-1-specific antibody (supershift). Cold competitors (shown only with c-Myc probe) included a wild-type (WT) probe or mutant probe lacking a mutated Blimp-1 binding site (Mut). Equivalent amounts of GST, GST-Ets1, or GST Δ331–415 (lacking the Ets domain) were used in the EMSA reactions. C, Western blot analysis of Blimp-1 levels in P3X lysates incubated with GST-Ets1 (or GST as a control). D, EMSA analysis performed as in A using the c-Myc binding site and P3X plasmacytoma extracts. Equivalent amounts of GST, GST-Ets1, GST-Ets2, or GST-PU.1 were added to the indicated reactions.

inhibition of Blimp-1 binding does not result from competition between GST-Ets1 and Blimp-1 for the same target site, as GST-Ets1 does not detectably bind to either the c-Myc probe or the Pax-5 probe (right-most lanes of Fig. 5, A and B). Importantly, incubation with GST-Ets1 did not affect the total amount of Blimp-1 protein found in P3X lysates, as demonstrated by Western blot analysis (Fig. 5C).

Given the fact that multiple Ets proteins were capable of interacting with Blimp-1 in the GST pulldown assay (Fig. 3D), we wished to determine whether they were all equally effective at inhibiting the binding of Blimp-1 to DNA. Thus, we incubated P3X lysates with increasing amounts of GST alone, GST-Ets1, GST-Ets2, or GST-PU.1 and monitored the Blimp-1 binding activity. Strikingly, GST-Ets1 alone among the different fusion proteins was capable of suppressing Blimp-1 DNA binding (Fig. 5D). This is particularly significant given the close amino acid sequence homology found in the Ets domains of Ets-1 and Ets-2. Interestingly, GST-PU.1, but not GST-Ets1 or GST-Ets2, bound to the c-Myc oligonucleotide probe. However, binding of GST-PU.1 to the probe did not inhibit Blimp-1 binding. Thus, our results indicate that Ets-1, but not other Ets family members, can interfere with the ability of Blimp-1 to bind to target DNA sequences.

Ets-1 Blocks the Repressive Function of Blimp-1—Because Ets-1 was capable of blocking DNA binding of Blimp-1, it might also affect the ability of Blimp-1 to repress target genes such as

the B cell-specific promoter PIII of the major histocompatibility complex class II transactivator *CIITA*, the *pax-5* (BSAP) promoter, and the *c-myc* promoter (10–12). To determine whether Ets-1 could interfere with the ability of Blimp-1 to repress these target genes, the A20 B cell line was transfected with luciferase reporter plasmids containing Blimp-1-dependent promoter segments along with constructs expressing Ets-1 and/or Blimp-1. Interestingly, for each of these promoters (*c-myc*, *pax-5* (BSAP), and *CIITA*), co-transfection with Ets-1 resulted in a dose-dependent activation of reporter gene expression (Fig. 6, A–C). This suggests that there are cryptic Ets binding sites located in each promoter, and indeed, the *c-myc* gene has previously been reported to be activated by Ets-1 (35).

Transfection with Blimp-1 repressed each of these promoters, consistent with previously published results (Fig. 6, A–C) (10–12). Co-transfection of Ets-1 relieved Blimp-1-mediated repression of the promoters in a dose-dependent manner (Fig. 6, A–C). This likely reflects both the ability of Ets-1 to directly bind these promoters and transactivate them as well as the ability of Ets-1 to interfere with Blimp-1 DNA binding. The ability of Ets-1 to alleviate Blimp-1-dependent repression of *pax-5* expression is intriguing given that down-regulation of *pax-5* is required for efficient differentiation into plasma cells (12, 19, 36, 37).

To determine whether the ability of Ets-1 to up-regulate the *pax-5* promoter in transient transfection assays correlated with an ability to up-regulate Pax-5 protein expression in B cells

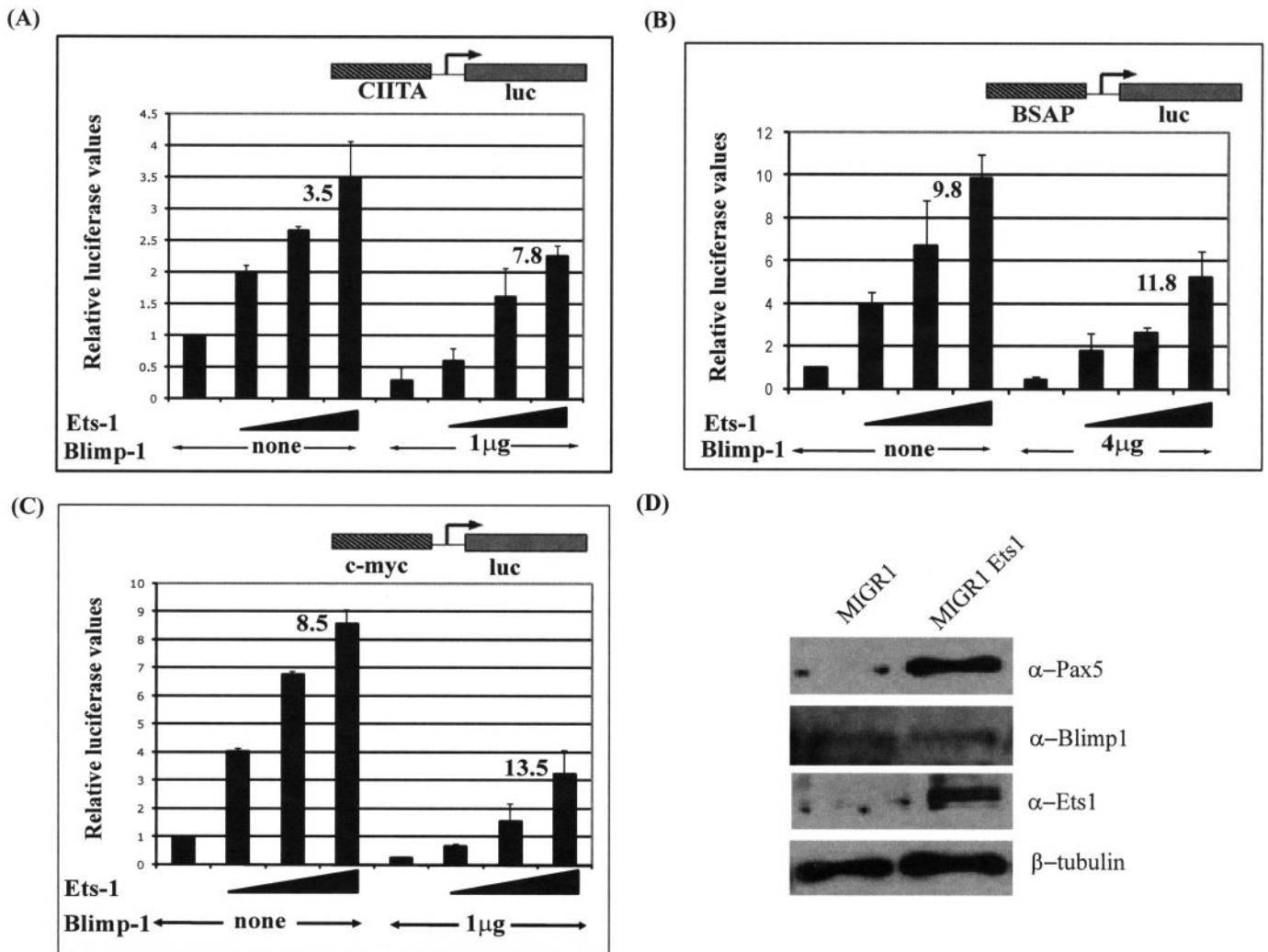


FIGURE 6. Ets-1 relieves Blimp-1-mediated repression of target genes. A20 B lymphoma cells were transfected with 5 μ g of CIITA-luc (A), BSAP-Luc (B), or c-MYC-Luc (C). Cells were co-transfected with 1 or 4 μ g of pCDNA3.1 Blimp-1 and varying concentrations of pCMV-HA-Ets1 (1, 2.5, or 5 μ g) as shown to determine the ability of Ets-1 and Blimp-1 to affect expression of these target genes. Cells were also co-transfected with 0.25 μ g of pCMV- β gal as an internal control. Shown are the averages of relative luciferase (*luc*) activities (after normalization to β -galactosidase) from three independent experiments. D, Western blot analysis of Ets-1, Blimp-1, Pax-5, and β -tubulin expression in sorted GFP⁺ cells from CpG-stimulated B cell cultures infected with MIGR1-Ets1 or the control virus (MIGR1).

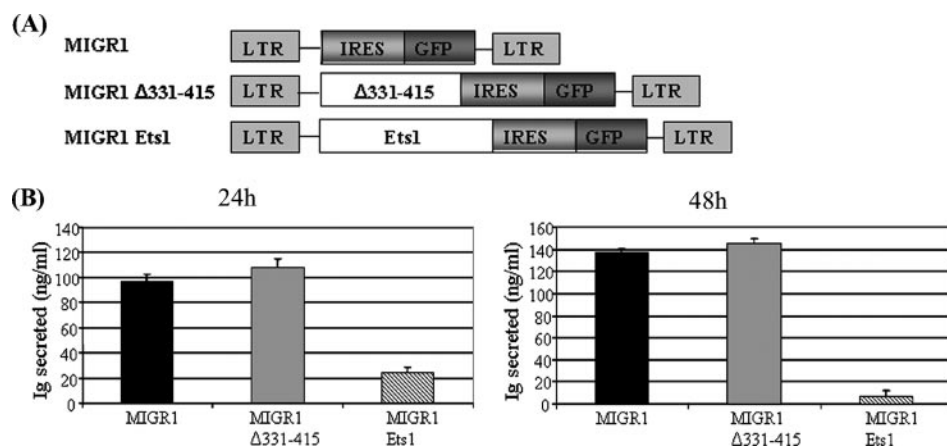


FIGURE 7. The Ets DNA binding domain is required for inhibition of IgM secretion. A, retroviral constructs used for transduction experiments. B, retroviral infections were carried out as described in Fig. 1. Two days post-retroviral infection, GFP-positive cells were sorted from each population and returned to culture in the presence of CpG ODN (10 μ g/ml). IgM secretion was analyzed 1 and 2 days thereafter by ELISA (representative results from one of three independent experiments are shown). LTR, long terminal repeat; IRES, internal ribosomal entry site.

induced to undergo differentiation, we sorted GFP⁺ cells from CpG-stimulated, retrovirally infected B cell cultures and assessed the levels of Ets-1, Blimp-1, and Pax-5 by Western blotting. As shown in Fig. 6D, enforced expression of Ets-1 driven by the retroviral vector did not alter the expression of Blimp-1 but led to strong up-regulation of Pax-5 expression. Hence, it is likely that Ets-1 functions in multiple ways to limit plasmacytic differentiation including blocking of Blimp-1 DNA binding as well as up-regulation of target genes normally repressed by Blimp-1, including the key B cell identity transcription factor *pax-5*.

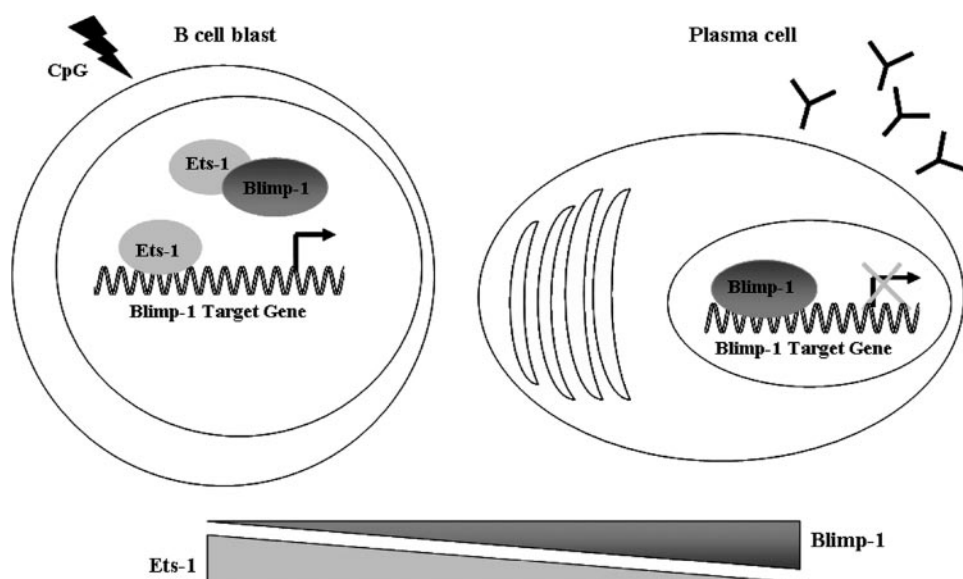


FIGURE 8. Proposed mechanism by which Ets-1 inhibits Blimp-1 function. The model hypothesizes that in stimulated B cells Ets-1 physically interacts with Blimp-1 to inhibit Blimp-1 DNA binding and thereby blocks repression of key Blimp-1 targets, thus preventing premature B cell differentiation. In addition, Ets-1 directly binds to the promoter segments of known Blimp-1 target genes (such as Pax-5) to up-regulate them (thus, counterbalancing the repressive influence of Blimp-1). Upon stimulation via TLR9, Ets-1 levels decrease relieving Blimp-1 of its inhibitory effects. Meanwhile, Blimp-1 levels increase, leading to efficient repression of its target genes and favoring terminal differentiation of the B cells.

Ets Domain of Ets-1 Is Required to Inhibit Plasmacytic Differentiation—The biochemical analyses we performed suggested that interaction of Ets-1 with Blimp-1 was mediated via the conserved Ets domain. In addition, the Ets domain of Ets-1 is required to bind to DNA sequences in the promoters and enhancers of target genes. Thus, we predicted that the Ets domain would be critical for the function of Ets-1 in blocking plasmacytic differentiation. To confirm this prediction, we generated a retrovirus expressing an Ets-1 mutant lacking the Ets domain (MIGR1 Δ 331–415) (Fig. 7A). Retroviruses containing the full-length or deleted forms of Ets-1 were used to infect purified splenic B cells stimulated with CpG ODN. Similar levels of full-length and deleted Ets-1 proteins were detected by Western blot (supplemental Fig. S3). The status of plasmacytic differentiation in the retrovirally infected populations was assessed by measuring IgM secretion from sorted GFP-positive cells. Enforced expression of full-length Ets-1 led to a significant reduction in IgM secretion over a 48-h period, similar to the results we obtained in Fig. 1 (Fig. 7B). In contrast, enforced expression of Ets-1 lacking the Ets domain did not affect the levels of IgM secreted, indicating that plasmacytic differentiation was not blocked by this protein (Fig. 7B). Thus, the Ets domain is critical for the function of Ets-1 in blocking plasmacytic differentiation.

DISCUSSION

Ets-1 Inhibits Blimp-1 Function in a B Cell-intrinsic Fashion—Previous studies have reported that mice lacking Ets-1 harbor an increased number of IgM-secreting plasma cells and increased serum IgM titers (20, 21). These observations suggest that Ets-1 plays a role in regulating the terminal differentiation of B cells into IgM-secreting plasma cells. Ets-1 is highly expressed in B cells, however, it has previously been unclear

whether the enhanced differentiation of Ets-1-deficient B cells is due to a B cell-intrinsic role for Ets-1 or, alternatively, to a non-B cell-intrinsic change in the micro-environment of the Ets-1 knock-out mouse.

Supporting a B cell-intrinsic role for Ets-1 is our observation that purified splenic B cells isolated from Ets-1-deficient mice (*Ets-1^{0/0}* mice) undergo increased terminal differentiation into IgM-secreting cells when cultured *in vitro* in the presence of CpG ODN (22). However, it was formally possible that the B cells purified from *Ets-1^{0/0}* mice had already been primed by unknown factors in the *Ets-1^{0/0}* microenvironment to have increased sensitivity to CpG ODN. In this report we demonstrate that enforced expression of Ets-1 in primary splenic B cells isolated from wild-type mice is sufficient to block efficient differentiation into CD138^{hi} plasma cells and secretion of

IgM (Fig. 1). These data confirm that Ets-1 functions in a B cell-intrinsic manner to limit differentiation of B cells into IgM-secreting plasma cells in response to signals downstream of TLR9.

Ets-1 Regulates B Cell Differentiation by Interfering with Blimp-1 Activity—To understand the mechanism by which Ets-1 regulates B cell differentiation in a cell-intrinsic manner, we focused our attention on a potential negative interaction between Ets-1 and the master regulator of plasmacytic differentiation Blimp-1. Blimp-1 is required to drive B cell terminal differentiation into immunoglobulin-secreting plasma cells by orchestrating repression of genes characteristic of mature B cells (38). We first demonstrated a reciprocal expression pattern between Ets-1 and Blimp-1. This result suggests that down-regulation of Ets-1 in addition to up-regulation of Blimp-1 may be essential for achieving effective plasmacytic differentiation. Importantly, however, there is a temporal window during B cell differentiation in which both Ets-1 and Blimp-1 are concurrently expressed and can potentially interact to regulate the differentiation process.

Several potential models can be envisioned to account for the ability of Ets-1 to inhibit plasma cell differentiation via an effect on Blimp-1. These include 1) binding of Ets-1 to regulatory sequences of the *blimp-1* gene leading to suppression of Blimp-1 expression (similar to the mechanism of action of Bcl-6 and Bach-2), 2) binding of Ets-1 to regulatory sequences in Blimp-1 target genes (e.g. *myc*, *CIITA*, *pax-5*) leading to up-regulation of these genes, and 3) direct protein-protein interaction between Ets-1 and Blimp-1 leading to inhibition of Blimp-1 function. Our data demonstrate that Blimp-1 expression is not down-regulated when high levels of Ets-1 are present in retrovirally infected B cells (Fig. 6D). In addition, Ets-1 does not inhibit the expression of a reporter gene construct in which the

blimp-1 promoter is fused to luciferase.³ However, the results presented in this report suggest that Ets-1 functions by both of the latter two models (up-regulation of Blimp-1 targets as well as binding the Blimp-1 protein) to limit plasma cell formation.

One of the Blimp-1 targets whose repression is up-regulated by Ets-1 is the crucial B cell identity transcription factor Pax-5. Pax-5 functions as both a transcriptional repressor, down-regulating genes inappropriate for mature B cell function (including genes characteristic of plasma cells), and as a transcriptional activator, up-regulating genes required to establish B cell identity (39). By promoting expression of Pax-5, Ets-1 could function to reinforce the commitment of cells to a mature B cell fate and inhibit their differentiation into plasma cells. Intriguingly, Ets-1 has also been shown to cooperate with Pax-5 to regulate the expression of Ig α (mb-1) (40, 41). Although it is unclear if Ets-1 and Pax-5 cooperate in regulating the expression of additional genes required for B cell identity, the existence of such a mechanism would allow these two transcription factors to form an interlinked regulatory network to reinforce the mature B cell fate and prevent plasmacytic differentiation.

In addition to its function in up-regulating the expression of Pax-5 and other genes important for B cell identity, Ets-1 also blocks plasma cell differentiation by its ability to directly interact with Blimp-1 and inhibit its function. We demonstrated this physical interaction in GST pulldown, co-immunoprecipitation experiments, and EMSA analyses. Interaction of Blimp-1 with Ets-1 inhibits its ability to bind target sequences, an effect that is dependent on protein-protein interactions, rather than competition for cognate DNA binding sites or from Ets-1-dependent degradation of Blimp-1. Based on these results, we propose a model (Fig. 8) where, after TLR9 stimulation of B cells, the relative concentrations of Ets-1 and Blimp-1 govern plasma cell formation. Early in the differentiation process, Ets-1 levels are high, and Blimp-1 levels are low, favoring the assembly of an Ets-1-Blimp-1 complex in which Blimp-1 is prevented from binding to its cognate DNA sequence. In addition, high levels of Ets-1 at this stage contribute to up-regulating the expression of genes such as Pax-5 that are repressed by Blimp-1. Later in differentiation Ets-1 levels fall, and Blimp-1 levels increase. Thus, Blimp-1 is relieved from the inhibitory effect of Ets-1, whereas activation of Blimp-1 target genes by Ets-1 is reduced. This allows effective repression of Blimp-1 target genes, thus driving B cell terminal differentiation.

The Ets Domain of Ets-1 Is Required for Its Interaction with Blimp-1—The interaction of Ets-1 with Blimp-1 was shown to depend on the Ets domain. Indeed, other Ets family proteins (Ets-2 and PU.1), both of which contain a similar Ets domain, were also capable of interacting with Blimp-1 in GST pulldown assays. Although Ets-1 and Ets-2 are virtually identical in amino acid sequence in the Ets domain, they are distinct in that only Ets-1 can inhibit Blimp-1 DNA binding. One potential model to explain this result is that the Ets domains of these proteins mediate the interaction but that sequences outside of the Ets domain (which are less well conserved between Ets-1 and Ets-2) are needed to interfere with Blimp-1 DNA binding activity.

In addition to its unique ability to block Blimp-1 DNA binding, Ets-1 might also have a special role to play in up-regulating target genes that are normally repressed by Blimp-1. Although all Ets proteins bind to a similar GGA(A/T) core sequence, differences in flanking sequences can govern the specificity of which Ets factor binds to a given site (42). Thus, the Ets binding motifs present in the promoter regions of Blimp-1 target genes might have a higher affinity for Ets-1 than for other Ets family members. It is also possible that Ets-1, but not other Ets proteins, efficiently cooperates with additional transcription factors that bind to these promoters, thus leading to a preferential role for Ets-1 in up-regulating these targets. Further studies will be necessary to determine the relative roles of Ets-1 and other Ets family members in regulating the expression of Blimp-1 target genes.

In summary, our data provide a new paradigm for the regulation of B cell terminal differentiation in response to TLR9 activation by the physical interaction of the key lineage-commitment protein Blimp-1 with a transcription factor that blocks its activity, Ets-1. This interaction is mediated by the conserved Ets domain of Ets-1, and indeed, other Ets factors are capable of interacting with Blimp-1 in GST pulldown assays, although they cannot inhibit Blimp-1 DNA binding activity. Ets-1 also functions to up-regulate a panel of target genes that are normally repressed by Blimp-1, including the crucial B cell identity factor Pax-5. Hence, it is likely that the relative concentrations of Ets-1 and Blimp-1 during plasma cell development play a critical role in determining the progression of the differentiation process.

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Shinu A. John, James L. Clements, Lisa M. Russell and Lee Ann Garrett-Sinha

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