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

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

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 knockout 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.

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-

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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.
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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, thereby contributing to and inactivating the key transcription factor regulating this decision, 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 Δ331–415. 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 Δ350–557) 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 μM 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 EF1α 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 μg/ml synthetic TLR9 ligand, CpG ODN 1826 (5′-TCCA-GACGTTCCTGACGTT-3′) (Coley Pharmaceuticals, Wellesley, MA), a phosphorothiate 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 (clone 3H2-E8, Novus Biologicals, Littleton, CO or clone 6D3, Santa Cruz Biotechnology), mouse 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 polylisine-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
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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 Assays**—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 mM Na3P2O7, 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 mouse monoclonal anti-FLAG antibody (clone M2, Sigma-Aldrich), or a control mouse IgG1 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 pCLeco plasmid using FuGENE 6 (Roche Applied Biosciences). Retroviral supernatants were used to infect 3 × 10^6 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<sup>bi</sup> and GFP<sup>pi</sup> populations were independently sorted. Proliferation of sorted GFP<sup>+</sup> 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 (clone11E10, 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<sup>−/−</sup>* 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...
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(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. β-tubulin serves as a loading control.

(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 quantify 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, GFPlo and GFPhi populations of virally infected cells were sorted as described in C above. IgM secretion was measured by ELISA 48 h after sorting. Representative results from one of two independent experiments are shown.

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 quanitate 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, GFPlo and GFPhi 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−CD138hi 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 (supplementary 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 CD138hi 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 GFPlo and GFPhi 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-
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Ets-1 Values were down-regulated during differentiation, we purified B220<sup>−</sup>CD138<sup>−</sup> B cell blasts and B220<sup>−</sup>CD138<sup>+</sup> 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<sup>−</sup> and CD138<sup>+</sup> cells. However, Ets-1 levels are ~8-fold higher in CD138<sup>−</sup> B cell blasts than in CD138<sup>+</sup> plasmablasts. Blimp-1 expression exhibits an opposite pattern being ~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 cell, 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 <sup>35</sup>S-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-
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/H9004\_54–135), the transactivation domain (GST/H9004\_136–242), and the N-terminal autoinhibitory domain (GST/H9004\_280–331) (Fig. 3C). In contrast, a GST-Ets1 fusion protein lacking the Ets domain (GST/H9004\_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/H9004\_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 pulldown 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 pulldown assays (supplemental Fig. S2). As shown in Fig. 3D, radiolabeled Blimp-1 interacted equivalently with all three Ets proteins, strongly supporting the functional significance 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/H9004\_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/H9004\_350–557) because we found Blimp1/H9004\_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 with the 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 (Δ331–415) failed to bring down Blimp-1 (lane 4, Fig. 4B). Importantly, reverse immunoprecipitations using anti-FLAG antibody showed a specific pulldown of Ets-1, whereas Ets-1 was undetectable when immunoprecipitated using the control mouse IgG antibody (Fig. 4B).
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.

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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 plasma-cytoma 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 Δ331–415 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 Δ331–415 had no affect on the complex (Fig. 5, A and B). The inability of the GST Δ331–415 protein (lacking the Ets domain) to block Blimp-1 DNA binding further confirms the results obtained in the GST pull-down assays and reiterates the functional importance of the Ets domain in mediating an effective interaction with Blimp-1. The
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. 5 C).

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.
induced to undergo differentiation, we sorted GFP/H11001 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 6.** Ets-1 relieves Blimp-1-mediated repression of target genes. A20 B lymphoma cells were transfected with 5 µg of CIITA-luc (A), BSAP-Luc (B), or c-MYC-Luc (C). Cells were co-transfected with 1 or 4 µg of pCDNA3.1 Blimp-1 and varying concentrations of pCMV-HA-Ets1 (1, 2.5, or 5 µg) 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 µg 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 µg/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.
Ets-1 Blocks the Function of Blimp-1

**DISCUSSION**

**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 Δ331–415) (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.

**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<sup>p/p</sup> 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<sup>p/p</sup> mice had already been primed by unknown factors in the Ets-1<sup>p/p</sup> 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<sup>hi</sup> 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. 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 and repressing the expression of Blimp-1) 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 both as 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 Igα (mb-1) (40, 41). Although it is unclear if Ets-1 and Pax-5 cooperate in regulating 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 member 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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