Early Growth Response-1 (EGR-1) and Nuclear Factor of Activated T Cells (NFAT) Cooperate to Mediate CD40L Expression in Megakaryocytes and Platelets*

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Background: Platelets mediate inflammatory activity through release of CD40L.

Results: CD40L expression in platelets is regulated by NFATc2 and EGR-1 activation in megakaryocytes.

Conclusion: Platelet inflammatory activity is dynamically regulated by exogenous factors that control NFAT and EGR-1 activity in megakaryocytes.

Significance: The NFAT/EGR-1 axis in megakaryocytes may be a new target for treatment of chronic inflammatory diseases.

Increasing evidence implicates circulating platelets as mediators of chronic inflammatory and autoimmune diseases via the expression and release of CD40L, an important modulator of inflammation and adaptive immune responses traditionally associated with activated T cells. Emerging evidence suggests that platelet CD40L is dynamically regulated in several chronic inflammatory and autoimmune diseases and may mediate progression and secondary pathology associated with those disease states. The present study identifies NFATc2 as a key transcriptional regulator of CD40L expression in megakaryocytes and inflammatory activity of platelets. Furthermore, the current data show that EGR-1, a member of the early growth response family of zinc finger transcription factors, modulates NFATc2-dependent regulation of CD40L expression in megakaryocytes. Our novel demonstration that in vivo biochemical or genetic inhibition of NFATc2 activity in megakaryocyte diminishes platelet CD40L implicates the NFATc2/EGR-1 axis as a key regulatory pathway of inflammatory and immunomodulatory activity in platelets and represents a target for the development of therapeutics for the potential treatment of chronic inflammatory and autoimmune diseases.

Beyond hemostasis, platelets are known to play a role in mediating inflammatory and immune responses via expression and release of proinflammatory factors, including CD154 (CD40L). CD40L is a type II protein ligand member of the tumor necrosis factor (TNF) superfamily that, via interaction with CD40, is a key immunomodulatory factor responsible for modulating nearly all aspects of the adaptive immune response. CD40L/CD40 interaction is required for enhancing antigen-presenting functions of dendritic cells, macrophages, and B cells; maturation of humoral responses; and enhancement of effector T cell responses (1). Additional functions of CD40L include the initiation of inflammatory and procoagulatory responses in vascular endothelial cells (2–5). The emerging role of CD40L in immunity-associated inflammation implicates CD40L as a key player in cardiovascular disease and several chronic autoimmune inflammatory diseases that target the vasculature, including systemic lupus erythematosus, diabetes, and chronic kidney disease (6–12). Subsequent to the discovery by Henn et al. (5) that platelets express and release biologically active CD40L capable of modulating CD40-dependent inflammatory activity in target cells, platelet CD40L is now implicated in the initiation, progression, and secondary pathology associated with many chronic inflammatory and autoimmune diseases. In light of these findings, the present study sought to understand mechanisms of CD40L regulation in platelets as a possible therapeutic target for the treatment of the aforementioned diseases.

It was established previously that platelet CD40L is derived from megakaryocytes and regulated via calcium mobilization-dependent activation of nuclear factor of activated T cells (NFAT), a family of calcium-dependent transcription factors most commonly associated with cytokine expression in activated T cells (13). Although each of the four calcium-dependent NFAT family members (NFATc1, -c2, -c3, and -c4) is expressed in T cells and has apparently redundant function, NFATc1 and NFATc2 are considered principle regulators of cytokines in activated T cells (14, 15). Outside the lymphoid compartment, NFAT is widely but variably expressed, and it is shown to mediate tissue-specific gene regulation during embryonic development and transduce inflammatory signals in endothelial cells (15–20). The nearly ubiquitous expression of the NFAT in diverse cell types suggests that NFAT protein is a key modulator of inflammation and adaptive immunity and has impact on human disease (18).

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The abbreviations used are: NFAT, nuclear factor of activated T cells; EGR, early growth response; BAPTA-AM, 1,2-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetracetic acid, tetraacetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; CsA, cyclosporine A; Z, benzoxylcarbonyl; fmk, fluoromethyl ketone; I–P, ionomycin plus PMA; EBS, Ets transcription factor family binding site.

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Although intracellular calcium flux is sufficient for nuclear translocation, NFAT weakly binds to its recognition site and requires additional co-factors for transcriptional activity (18). Recently, specific members of the early growth response (EGR) transcription factor gene family, notably EGR-1, were identified as factors required for NFAT-dependent CD40L expression in activated T cells (21, 22). EGR-1 has also been associated with megakaryocytic development and the regulation of Gqα, a megakaryocyte-specific gene associated with signal transduction in platelets (23–25). The observation that EGR-1 is expressed in megakaryocytes and modulates NFAT-dependent expression of CD40L in T cells prompted the investigation of EGR-1 as a potential modulator of NFAT-dependent expression of CD40L in megakaryocytes and, hence, platelet inflammatory activity.

The current study further characterizes the mechanism of NFAT-mediated regulation of CD40L and shows that EGR-1 is required for NFAT-mediated expression of CD40L in megakaryocytes. It was determined that NFATc2 specifically modulates CD40L transcription and that EGR-1 is required for NFATc2 binding and transactivation of the CD40L promoter. Importantly, biochemically inhibiting NFAT activity or abrogating NFATc2 expression in vivo significantly decreased CD40L mRNA expression in bone marrow megakaryocytes and CD40L protein in circulating platelets. These data show for the first time that modulation of upstream regulatory mechanisms of CD40L expression in megakaryocytes can impact inflammatory activity of platelets and suggest that NFATc2 and EGR-1 activity may be potential therapeutic targets for the modulation of platelet-mediated inflammation in disease.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—The CMK, K562, and Jurkat cell lines were purchased from ATCC (Manassas, VA) and were routinely maintained in RPMI 1640, supplemented with 10% heat-inactivated FCS, 10 mM-glutamine, and 100 mg/ml penicillin/streptomycin at 37 °C and 5% CO2. 1,2-bis-(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM), phorbol 12-myristate 13-acetate (PMA), ionomycin, and cyclosporine A (CsA) were all purchased from Sigma. Injectable CsA was purchased from Bedford Laboratories (Cleveland, OH). The pan-caspase inhibitor Z-VAD-fmk was purchased from BioMol (Plymouth Meeting, PA).

Megakaryocyte differentiation of the K562 and CMK cell lines were performed as described (13). Briefly, CMK cells were cultured in complete medium with 10% normal fetal calf serum (FCS), 10% heat-inactivated FCS, 10 mM-glutamine, and 100 mg/ml penicillin/streptomycin at 37 °C and 5% CO2. 1,2-bis-(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM), phorbol 12-myristate 13-acetate (PMA), ionomycin, and cyclosporine A (CsA) were all purchased from Sigma. Injectable CsA was purchased from Bedford Laboratories (Cleveland, OH). The pan-caspase inhibitor Z-VAD-fmk was purchased from BioMol (Plymouth Meeting, PA).

Mice—Normal C57BL/6 (B6) and BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained as a breeding colony. The NFATc2 homozygous knock-out mice (NFATc2null) on a BALB/c background were a generous gift from Laurie Glimcher (Harvard University, Boston, MA) (26). All animals were maintained at the Purdue University Animal Care Facility, and all animal experiments followed approved Institutional Animal Care and Use Committee and Institutional Review Board protocols. To generate NFATc2null on a C57BL/6 (B6) background, NFATc2−/− mice were backcrossed with wild type B6 mice for 13 generations then crossed with heterozygous littermates to generate B6 NFATc2−/−.

**Transient Transfections and Luciferase Assays**—K562 and CMK cells were transfected using Lipofectamine 2000 reagent (Invitrogen) using the manufacturer’s protocol. Each transfection used 3.2 μg of reporter plasmid along with 0.5 μg of pSv-βGal as a transfection efficiency control plasmid. Twenty-four hours after transfection, the cells were lysed in 200 μl/well of reporter lysis buffer (Promega, Madison, WI). Luciferase activity was determined by mixing the lyssate with luciferase substrate (Promega) and immediately reading the sample in a Monolight 2010 luminometer (BD Biosciences). β-Galactosidase activity was determined using Galactolylte β-galactosidase substrate (Tropix, Bedford, MA) and reading the sample in the luminometer. Luciferase activity was normalized by dividing the mean luciferase relative light units by the mean β-galactosidase relative light units.

**Real-time RT-PCR**—Total cellular RNA was isolated from all cells using the RNAeasy kit (Qiagen, Valencia, CA), and cDNA synthesis was performed using Superscript III reverse transcriptase (Invitrogen), following the manufacturer’s instructions. Diluted cDNA was subjected to real-time quantitative PCR (qRT-PCR) using proprietary TaqMan primer and probe sets for human and mouse CD40L, EGR1, Fli-1, NFATc1, NFATc2, NFATc3, NFATc4, MCP-1 mRNA, and 18 S rRNA purchased from Applied Biosystems (Foster City, CA) and Perfecta qPCR Supermix (Quanta Biosciences, Gaithersburg, MD). For each sample, three PCRs were performed. The resulting relative increase in reporter fluorescent dye emission was monitored by the TaqMan system (GeneAmp 5700 sequence detection system and software, PerkinElmer Life Sciences). The level of test mRNA, relative to 18 S rRNA, was calculated using the formula, relative mRNA expression = 2−(Ct(test)−Ct(18 S rRNA)), where Ct is the threshold cycle value.

**Plasmid Constructs**—The p-391wt, p-391DNFATmut, and p-391PNFATmut CD40L promoter-luciferase reporter constructs have been described previously (13). The p-391EBSmut and p-391NFATKOmut vectors were produced using PCR-based site directed mutagenesis and subcloning of the modified CD40L promoter region into the pGL-3 basic luciferase reporter vector (Promega) as described previously (13). The EGR-1 expression vector, obtained from the Addgene plasmid repository (Addgene plasmid 11729) was described previously and was a generous gift from Eileen Adamson (27). The NFATc3 expression vector (Addgene plasmid 11790) was a generous gift from Anjana Rao (Harvard University). The NFATc2 expression vector was produced by cloning the
NFATc2 cDNA from pENTR11 (Addgene plasmid 11791, also a gift from Anjana Rao) into pcDNA3.1 (Invitrogen).

Generation of CMK Megakaryocytes with Stable EGR-1 Overexpression—CMK cells were transfected with the EGR-1 expression vector (EGR1 cDNA in the pcDNA3.1 vector backbone) or pcDNA3.1 only using Lipofectamine 2000 according to the manufacturer’s instructions. Stable clones were isolated after a 14-day culture in medium containing 400 mg/ml G418 (Genetecin, Life Technologies). Neo-resistant colonies were cultivated and assessed for EGR-1 expression in the cell lysates by EGR-1 ELISA using anti-EGR-1 monoclonal and polyclonal antibodies purchased from Abcam (Cambridge, MA). The CMK clone c4 (CMK(EGR-1)c4) shows high level EGR-1 expression (data not shown) and was further cultivated for use in the experiments described in the legend to Fig. 6.

Electrophoretic Mobility Shift (EMSA) and Supershift Assays—Nuclear extract was prepared from PMA-differentiated CMK cells treated with 1+P for 4 h as described previously (13). Oligonucleotides corresponding to the sense (5’-end-labeled with biotin) and antisense strands of each probe (90 μM) were synthesized by Integrated DNA Technologies (Coralville, IA) and annealed by heating at 95 °C for 10 min, followed by slow cooling to room temperature. The identities and sequences of the biotin-labeled oligonucleotide probes and the non-labeled competitor/non-competitor oligonucleotides are shown in Fig. 4.

EMSA and supershifting was performed using the LightShift Chemoluminescent EMSA kit from Pierce according to the manufacturer’s instructions. Briefly, 20 fmol of biotin-labeled probe was incubated with nuclear extract (2.5 μg of total protein) for 20 min at room temperature in binding buffer with 5 mg/ml poly(dI-dC). Protein-DNA complexes were subjected to electrophoresis through 6% DNA retardation gels (Invitrogen) in Tris borate, EDTA (TBE) buffer for 1–2 h at room temperature and then transferred to Biodyne® B positively charged nylon membrane (Pall, Port Washington, NY). Thereafter, detection was carried out using the Chemiluminescence Nucleic Acid Detection Module (Pierce), as described by the manufacturer. The membrane was visualized on a UVP Chemi-Darkroom and analyzed with VisionWorks software (Upland, CA). In binding reactions containing competitor oligonucleotides, 200-fold molar excesses (4 pmol) of non-labeled double-stranded competitors/non-competitors were added for 20 min before electrophoresis.

For supershift assays, nuclear extract (2.5 μg of total protein) was preincubated with 3 μg of anti-NFATc1, anti-NFATc2, anti-NFATc3, anti-EGR-1, anti-Fli-1, or normal rabbit IgG (IgCon) (all purchased from Santa Cruz Biotechnology, Inc.) for 20 min at 4 °C. Thereafter, the nuclear extract/antibody mixtures were incubated for 20 min at room temperature with the biotin-labeled probe before electrophoresis and detection as described.

Chromatin Immunoprecipitation (ChIP) Assays—The preparation of chromatin from differentiated and undifferentiated CMK cells is described in the supplemental material. ChIP was performed using monoclonal antibodies to NFATc2 (sc-7296, mouse IgG2a; Santa Cruz Biotechnology), NFATc1 (sc-7294, mouse IgG1; Santa Cruz Biotechnology), or EGR-1 (sc-189, rabbit polyclonal IgG; Santa Cruz Biotechnology) and agarose-Protein A/G beads (Santa Cruz Biotechnology). Control precipitations were performed using isotype control antibodies (for NFATc1 and -c2) or non-immune rabbit IgG (for EGR-1), all purchased from Santa Cruz Biotechnology. Immunoprecipitated DNA eluted from protein A/G beads was amplified by quantitative PCR using a Chromo4 real-time PCR detection system (Bio-Rad) and dual-labeled fluorescent probe sets (IDT) to the distal NFAT and proximal NFAT/Ets transcription factor family binding site (EBS) in the CD40L promoter. Probe sets with the indicated fluorescent dye (FAM) and quenchers (ZEN/Iowa Black FQ) used are described in the supplemental Materials and Methods.

Generation and Validation of the EGR1 shRNA Constructs—The generation of the EGR1 shRNA knockdown vectors was performed using the BLOCK-iT lentiviral RNAi expression system (Invitrogen) according to the manufacturer’s instructions. Briefly, the selection of shRNA sequences was based on Web-based shRNA design software (Invitrogen). Candidate sequences were analyzed for EGR1 mRNA specificity using the BLAST. Four individual sequences were selected for testing. The sense and antisense oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA) and were annealed and cloned into the BLOCK-iT U6 RNAi entry vector according to the manufacturer’s instructions (Invitrogen). The U6-RNAi cassette was then transferred into pLenti6/BLOCK-iT-DEST promoterless lentiviral vector by LR clonase-mediated recombination (Invitrogen). The pLenti6 shRNA vectors containing the candidate sequences were transiently transfected into K562 cells and then monitored for endogenous EGR1 mRNA expression knockdown by qRT-PCR as described under “Experimental Procedures.” The 1508 sequence (5’-GGCATACCAAGATCCACTTG-3’) was shown to suppress EGR1 mRNA to less than 35% of normal expression in K562 cells and results in an 82% reduction of endogenous EGR1 protein by ELISA (supplemental Fig. S1). A second pLenti6 U6 construct was then produced, consisting of the 1508 sequence scrambled to serve as a shRNA control.

Platelet Isolation and CD40L ELISA—Mice were anesthetized and bled by cardiac puncture. Blood was collected into syringes containing 1.0 ml of acid/citrate/dextrose (12.5 g/liter sodium citrate, 10.0 g/liter d-glucose, and 6.85 g/liter citric acid), added to 6 ml of PIPES buffer (150 mM NaCl and 20 mM PIPES (pH 6.5)), and spun at 100 × g for 15 min. The platelet-rich supernatant was collected, and 1 unit/ml apyrase and 1 μM prostaglandin E1 (final concentrations) (Sigma) were added and spun at 1000 × g for 10 min. The platelet pellet was washed once in Tyrode’s buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na2PO4, 12 mM NaHCO3, 20 mM HEPES, 1 mM MgCl2, 5 mM glucose, and 0.5 mg/ml bovine serum albumin, pH 6.5), repelleted, and then lysed in phosphate-buffered saline (PBS) with 0.1% Triton X-100 for 10 min on ice. Insoluble material was removed by centrifugation. Total protein analysis of the platelet lysates was performed using the BCA protein assay kit (Pierce). All cleared lysates were stabilized by the addition of protease inhibitor mixture (Sigma) and stored at −80 °C until analysis. CD40L protein expression in the platelet lysates was determined by ELISA using the mouse sCD40L ELISA kit.
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(BMS6010, Bender Medsystems) according to the manufacturer’s protocol. All samples were performed in at least triplicate, and absolute CD40L levels were normalized to total protein.

Statistical Analysis—All statistical analyses of data were carried out using GraphPad Prism (GraphPad Software Inc). The unpaired Student’s *t* test was used to compare experiments between two groups. Where noted, luciferase experiments were expressed as means ± S.E. of comparative -fold differences. Values were considered significant at a *p* value of < 0.05 (*), < 0.01 (**), and < 0.001 (***)

**RESULTS**

Calcium Mobilization and PKC Activation Rapidly and Potently Induce CD40L Expression in Primary Megakaryocytes—To verify CD40L induction in the absence of cytokines and growth factors that may alter calcium mobilization and/or PKC activation, the expression of CD40L mRNA was assessed in ionomycin and PMA-induced, freshly isolated primary megakaryocytes. Primary CD41+ megakaryocytes were isolated from total mononuclear bone marrow cells from normal C57Bl/6 mice, as described in the supplemental Materials and Methods. The purified CD41+ megakaryocytes were pretreated for 30 min in the presence of vehicle alone, CsA, or BAPTA-AM before the addition of ionomycin, PMA, or I+P for 4 h. The expression of CD40L mRNA was then assessed by quantitative RT-PCR (qRT-PCR). The data in Fig. 1A show that although ionomycin treatment alone was sufficient to induce levels of CD40L mRNA, I+P significantly enhanced expression in CD41+ megakaryocytes. The data also show that pretreatment with either the CsA or BAPTA-AM completely blocks I+P-induced expression of CD40L mRNA, indicating that calcium mobilization is required for induced expression in primary megakaryocytes. Similar results were obtained when we performed identical experiments using the megakaryocytic cell line CMK after PMA-induced terminal megakaryocytes differentiation (Fig. 1B), validating the use of the CMK cell line for the elucidation of inducible expression of CD40L in megakaryocytes. Overall, these data indicate the both calcium mobilization and PKC activation were required for maximal CD40L expression.

Both the Proximal NFAT Binding Site and the EBS Are Required for I+P-inducible CD40L Transcriptional Activity—We previously demonstrated that two consensus NFAT binding elements (core sequence GGAAAA), a distal site (DNFAT) located at −256 bp, and proximal NFAT site (PNFAT) at −57 bp upstream of the transcription start site of the CD40L promoter, play a key role in differentiation-mediated CD40L transcription in megakaryocytes (13). In addition to the NFAT binding sites, a consensus EBS immediately downstream from the proximal NFAT element was recently shown to be required for NFAT-dependent transactivation of CD40L in activated T cells (21). Because NFAT sites are often dependent on neighboring cis-acting elements for transcriptional function, and several Ets family transcription factors are shown to be important in the regulation of megakaryocyte-associated genes, the role of the EBS in the modulation of CD40L promoter activity was assessed in megakaryocytes (28–31). To map the I+P-responsive elements in the CD40L promoter, CMK cells were transfected with the wild type CD40L promoter-reporter construct or similar constructs containing specific mutations to the distal and/or the proximal NFAT sites or the EBS and then allowed to rest for 24 h before stimulation with I+P for 6 h. The data in Fig. 2 show deletion of the distal NFAT site only did not significantly decrease I+P-inducible transcriptional activity; however, deletion of either proximal NFAT site or the EBS alone abrogated I+P-inducible activity of the CD40L promoter. Furthermore, in the absence of both functional NFAT sites, an intact EBS is not sufficient for I+P-mediated transactivation. These data indicate co-regulation of CD40L transcription via both the EBS and proximal NFAT binding site.

**EGR-1 Occupies the EBS and Modulates NFATc2 Binding to the Proximal NFAT Site**—Given the dependence of functional EBS and proximal NFAT sites for I+P-inducible CD40L transactivation, megakaryocyte expression of NFAT and Ets transcription factors associated with these sites was next characterized. Previously, Kiani’s group (19, 32–34) demonstrated that...
the four NFAT proteins are differentially expressed during primary megakaryocyte differentiation and in several human megakaryocyte-like cell lines. To confirm similar expression in our laboratory, the relative mRNA expression of the four calcium-dependent NFAT family members were compared in megakaryocyte-differentiated K562 and CMK cells, unstimulated T cell lymphoma cell line, Jurkat, and freshly isolated CD41+/H11001 megakaryocytes by qRT-PCR (Fig. 3A). In agreement with the previous observations, the cell lines showed differential expression of NFAT. The relative levels of the different NFAT mRNAs were similar in the CMK, CD41+ megakaryocytes, and Jurkat cells, with NFATc3 expression being the highest, followed by NFATc2 and then NFATc1. Interestingly, despite an established role for NFATc1 and NFATc2 in activation-dependent expression of CD40L in T cells, CMK and primary CD41+ megakaryocytes both showed greater than 4-fold the levels of all three of the expressed NFAT proteins as compared with Jurkat T cells, suggesting that NFAT plays an equally important role in megakaryocyte gene expression.

Based on the promoter analysis, the putative requirement for an intact EBS suggests additional factors capable of binding to the EBS are important for I+P-induced CD40L transcription. We show that two candidate transcriptional factors known to be capable of binding to the EBS are expressed in megakaryocytic cells. Fig. 3B shows that Fli-1, an Ets family transcription factor previously shown to be required for megakaryocyte-specific gene expression, is basally expressed in CMK and CD41+ megakaryocytes and moderately expressed in unstimulated Jurkat cells. In contrast to the megakaryocytic or T cells, Fli-1 is feebly detectable in megakaryocyte-differentiated K562 cells. An additional candidate transcriptional regulator recently shown to be capable of interacting with the EBS is EGR-1, a
ubiquitous proliferation-associated transcriptional regulator previously shown to modulate NFAT-mediated CD40L in activated T cells via interaction with the EBS (21). As seen in Fig. 3C, EGR1 mRNA abundantly expressed in K562 cells while moderately and equally expressed in primary CD41/H11001 megakaryocytes and differentiated CMK cells. In contrast, Jurkat cells show relatively low basal expression as compared with the differentiated hematopoietic/megakaryocytic cell lines or primary CD41+/megakaryocytes.

To identify specific NFAT occupancy of the proximal NFAT binding site and to identify protein binding to the EBS, EMSA and supershifting was employed. Nuclear extracts prepared from I/H11001P-stimulated CMK cells were incubated with the labeled oligonucleotide probe containing the proximal NFAT element and putative EBS. Two complexes (I and II) are clearly detected bound to the labeled probe (Fig. 4A). Binding of complex I to the labeled probe was inhibited by a 200-fold addition of a non-labeled oligonucleotide containing a mutation of the EBS site (GGAA to GAGA) but an intact NFAT site (TTTTCC) (Fig. 4A, EBS Nonspecific comp). Binding of both complexes I and II were inhibited by a oligonucleotide containing both intact EBS and NFAT sites (Fig. 4A, EBS/NFAT Specific comp). Supershifting was performed by preincubating the nuclear extracts with antibodies to NFATc1, NFATc2, NFATc3, Fli-1, EGR-1, or the IgG control before binding reactions with the labeled probe. The addition of anti-NFATc2 caused a supershift of complex I (Fig. 4A, NFATc2) that was not evident by the addition of antibodies to NFATc1, NFATc3, or the IgG control, indicating that NFATc2 (complex I) preferentially occupies the proximal NFAT site. The addition of an antibody to EGR-1 (Fig. 4A, EGR-1) but not Fli-1 (Fig. 4, Fli-1) diminishes the binding of both complex I and II. These results indicate functional interaction between the EBS and the proximal NFAT site and suggest possible physical interaction between EGR-1 and NFATc2. To assess NFAT and EGR-1 binding to the proximal CD40L promoter in vivo, ChIP assays were performed. Chromatin was extracted from differentiated CMK cells, either treated with vehicle only (DMSO) or I/H11001P-stimulated for 2 h. Transcription factors bound to DNA were immunoprecipitated with specific antibodies or their respective isotype controls, directed against NFATc1, NFATc2, or EGR-1. Immunoprecipitated chromatin was amplified and quantified by real-time PCR using primers flanking either the distal NFAT site or the proximal NFAT/EBS site (Fig. 4B). In differentiated but non-I+P-stimulated CMK cells, immunoprecipitation with antibodies to both NFATc1 and NFATc2 enriched the distal promoter region, indicating that NFATc1 and NFATc2 are constitutively bound to the distal NFAT site, which increases after stimulation with I+P. In contrast to the distal NFAT site, NFATc2 and EGR-1 but not NFATc1 are only bound to the proximal NFAT/EBS site after I+P stimulation. Overall, these data demonstrate that NFATc1 and NFATc2 are constitutively bound to the distal NFAT site and, in agreement with our previous studies, play an important role in constitutive CD40L expression in megakaryocytes. NFATc2, however, is the dominant NFAT family member modulating inducible CD40L expression in megakaryocytes, and the EMSA data suggest that EGR-1 modulates NFATc2 binding to the proximal NFAT site.
EGR-1 Modulates NFATc2 Transcriptional Activity in the CD40L Promoter—Given the dependence on EGF-1 occupancy of an intact EBS for NFATc2 binding, it is hypothesized that EGR-1 was directly modulating NFATc2-mediated transcriptional activity of the CD40L promoter in megakaryocytes. To determine if ectopic expression of EGR-1 can modulate NFATc2 activity, expression vectors encoding NFATc2, NFATc3, or EGR-1, either alone or in combination, were cotransfected along with the CD40L promoter-reporter construct (p-391-Luc) into undifferentiated K562 or CMK cells. Twenty-four hours after transfection, cells were treated with a combination of I+P or vehicle only for 2 h followed by cross-linking and chromatin isolation and sonication as described under “Experimental Procedures.” Chromatin was immunoprecipitated with anti-Egr-1, NFATc1, and NFATc2, or their respective isotype control antibodies. After reverse cross-linking, immunoprecipitated chromatin was amplified and quantified by real-time PCR using primers flanking either the distal NFAT site or the proximal NFAT site + EBS. -Fold differences in binding relative to isotype antibodies were calculated (see “Experimental Procedures”), and averages and S.E. values from three replicate samples are depicted for each test transcription factor analyzed. The data shown are representative of three individual experiments. *, p < 0.05; **, p < 0.01.
four hours after transfections, luciferase activity was determined as before. Fig. 5 shows that in undifferentiated CMK cells, which in comparison with K562 cells express lower basal levels of endogenous EGR1 mRNA but much higher basal levels of NFATc2, overexpression of EGR-1 was sufficient to drive promoter activity ~6-fold over the empty vector control. In contrast to EGR-1, overexpression of NFATc2 or NFATc3 alone only slightly enhanced promoter activity in CMK cells. However, co-expression of both transcription factors resulted in synergistic activation of the CD40L promoter greater than 12-fold over controls. As indicated in Fig. 3, in contrast to CMK cells, K562 cells have nearly undetectable levels of basal NFATc2 mRNA expression and roughly 2 times the basal expression of EGR1 mRNA. In K562 cells, overexpression of NFATc2 alone, but not NFATc3 nor EGR-1 alone, was sufficient to enhance CD40L promoter activity more than 10-fold. Overexpression of both EGR-1 and NFATc2 only slightly enhanced additional promoter activity over K562 cells overexpressing EGR-1 alone. The differential responses to co-

**ERG-1 Modulates Endogenous CD40L Expression**—The transient transfection studies demonstrated that ectopic overexpression of EGR-1 was capable of modulating NFATc2-dependent regulation of the CD40L promoter. To verify that overexpression of EGR-1 augments endogenous CD40L mRNA expression, CMK cells were stably transfected with vector and clones with high level EGR-1 expression (supplemental Materials and Methods and Fig. S1) and then assayed for CD40L expression after 6 h of I+P stimulation by RT-PCR. Interestingly, our initial experiments with the stable CMK clones showed significant loss in cell viability after I+P stimulation (supplemental Materials and Methods and Fig. S2), leading us to postulate that overexpression of EGR-1 may also be inducing the expression of proapoptotic genes. Thus, to determine if EGR-1 augments endogenous CD40L, the CMK clone (CMK(Egr-1)c4) was induced in the presence of apoptosis inhibitor Z-VAD-fmk before and during I+P stimulation. As expected and as shown in Fig. 6A, overexpression of EGR-1 enhances I+P-mediated expression of endogenous CD40L mRNA only in the presence of Z-VAD-fmk. Although these data confirm that overexpression of EGR-1 can enhance endogenous CD40L and proapoptotic genes, it is not clear from these studies if modulation of endogenous EGR-1 can regulate I+P-induced CD40L expression. Thus, to determine the impact of endogenous EGR-1 modulation on CD40L in megakaryocytes, EGR1 mRNA-specific shRNA (or scrambled matched control) constructs were transiently transfected into CMK cells and then assessed for CD40L expression with and without I+P stimulation (for design, construction, and testing of the shRNA constructs, see supplemental Materials and Methods and Fig. S1). We first confirmed the ability of the EGR1 mRNA-specific shRNA sequence (shRNA 1508) versus the scrambled control (1508 scramble) to knock down endogenous EGR1 mRNA by transfection of K562 cells, previously shown to constitutively express high levels of EGR-1 (supplemental Fig. S1). As shown in Fig. 6B, transfection of CMK cells with shRNA 1508 (NFAT KO) decreased endogenous EGR1 mRNA expression to less than 45% of the empty vector (Mock) or 1508 scrambled vector (Control) in I+P-stimulated CMK cells. Likewise, endogenous CD40L mRNA was reduced by similar levels (~45%) in CMK cells with shRNA-mediated knockdown of endogenous EGR-1. Overall, these results confirm a regulatory role for EGR-1 in NFAT-mediated expression of CD40L in megakaryocytes.

**Systemic or Genetic Down-modulation of NFAT Activity Diminishes Megakaryocyte and Platelet CD40L**—The in vitro analysis of the CD40L regulation in megakaryocytes allowed for the identification of possible upstream pathways of CD40L expression in platelets. It was hypothesized from these data that systemic, in vivo modulation of megakaryocyte NFAT activity in general and NFATc2 activity specifically would impact CD40L protein levels in platelets. Because platelet-associated
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CD40L is regulated and expressed by megakaryocytes and later transported into platelets, we assumed that systemic inhibition of CD40L transcription in megakaryocytes during maturation before platelet release was required to potentially observe changes in circulating platelet expression. Therefore, to determine effects of systemic inhibition of NFAT activity on platelet inflammatory activity, normal C57B/6 mice were treated daily for 14 days with CsA at 100 mg/kg/day by intraperitoneal injection. Control mice were given daily sham injections of vehicle only (1:1 (v/v) mineral oil/PBS). In addition to pharmacological inhibition of NFAT activity, the impact of specific NFATc2 inhibition on megakaryocyte and platelet CD40L expression was also assessed in NFATc2 knock-out mice (26). The data shown in Fig. 7A confirm diminished expression of CD40L in freshly isolated primary CD41+ megakaryocytes from both the CsA-treated and NFATc2 knock-out mice and confirm that CD40L expression in megakaryocytes from NFATc2 knock-out mice is refractory to I+P stimulation. Although basal CD40L expression was diminished in the freshly isolated CD41+ cells isolated from the CsA-treated mice, CD40L mRNA was induced by I+P stimulation. The ability of megakaryocytes to respond to I+P ex vivo is most likely due to the fact that NFAT activity is no longer inhibited by systemic CsA once isolated from the bone marrow. CD40L protein levels were also assessed in platelets isolated from similarly treated mice (Fig. 7B), correlating with diminished CD40L expression in megakaryocytes. Overall, these data support the hypothesis that interruption of the modulation of upstream regulatory pathways in megakaryocytes can ultimately impact proinflammatory activity of platelets and suggest that NFATc2 and EGR-1 transcriptional pathways are potential therapeutic targets that can modulate platelet-mediated inflammation.

DISCUSSION

The emerging role of platelets as modulators of inflammation and adaptive immune responses and the association of platelet-mediated inflammation in several diseases prompted investigation of CD40L regulation in megakaryocytes and platelets. NFAT transcription factors are classically associated with inducible expression of several TNF family proteins, including CD40L and FasL, in activated T cells (26). Although NFAT proteins have widespread expression in different tissue types, little is known about potential target genes in non-lymphoid cells. NFATc1 and -c2 were shown to regulate vascular endothelial cell growth factor (VEGF)-mediated inflammatory responses in human endothelial cells (35). The same NFAT proteins were also shown to play an important role in osteoblast differentiation during embryonic development (36). Kiani and associates (19, 32) first demonstrated a potential role of NFAT-mediated gene expression in the megakaryocyte compartment by demonstrating distinct expression profiles of the NFAT proteins during myeloid lineage development. Subsequently, the same group showed NFATc2 mediated the expression of FasL, a proapoptotic and immunomodulatory gene thought to be important during the process of thrombopoiesis in megakaryocytes (32, 34). Recently, they showed direct evidence that NFAT regulates apoptosis in megakaryocytes via regulation of the FasL/Fas pathway (33). Our demonstration that overexpression of EGR1 with concomitant activation of NFAT induced cell death in differentiated CMK (Fig. 6) suggests that EGR-1 may cooperate with NFAT to further enhance NFAT-mediated induction of FasL as well as CD40L.

Comparative analysis of the differentiated CMK and primary CD41+ megakaryocytes reveals similar basal expression levels of three of the four NFAT family members (NFATc1–3). The present study showed that despite redundant function among
the different NFAT proteins and the observation that NFATc3 is the most abundantly expressed NFAT and that NFATc1 plays a regulatory role in constitutive CD40L expression, only NFATc2 occupies the proximal NFAT site in the CD40L promoter after I/II1001P stimulation. Furthermore, only overexpression of NFATc2 induces CD40L transactivation and expression of endogenous CD40L. Therefore, like T cells, the current data support the notion of megakaryocyte-specific utilization of the functionally similar NFAT transcription factors.

Because calcium-mediated NFAT translocation to the nucleus is not sufficient to induce transactivation of NFAT target genes, the current paradigm is that additional signals, such as activation of mitogen-activated kinases (MAPKs), are required to drive NFAT transcriptional activity (37–40). Consistent with this paradigm, both ionomycin and PMA signaling are required for optimal CD40L induction in megakaryocytes. The requirement for a second signal in addition to calcium mobilization has prompted investigations into identifying cofactors that can modulate NFAT activity as targets for the pharmacological modulation of NFAT target genes. EGR-1 is member of a family of immediate early response proteins rapidly and transiently induced by several stimuli, including growth factors, cytokines, and cell stress. Several EGR transcription factors modulate NFAT activity via binding to consensus EGR binding elements proximal to NFAT binding sites in the promoters of interleukin-2 (IL-2), TNFα, and FasL in activated T cells (22, 41, 42). Ironically, the proximal CD40L promoter lacks any consensus EGR binding elements (MatInspector transcription factor binding site motif database). However, recently, the consensus EBS was shown to alternatively bind EGR-1 and modulate transcription of CD40L in activated T cells (21). The present studies show that despite abundant expression of FlI-1, an Ets family transcription factor associated with modulation of megakaryocyte-associated gene expression, EGR-1 occupancy of the EBS is required for NFAT-mediated transactivation of CD40L in megakaryocytes and suggests that EGR-1 apparently mediates NFATc2 activity by stabilizing NFATc2 binding to the proximal binding site (30, 43).

The novel demonstration that in vivo inhibition of NFAT activity with CsA (or by genetic knockout of NFATc2) impacts CD40L-dependent inflammatory activity of platelets shows that platelet CD40L can be dynamically regulated by upstream transcriptional regulators in megakaryocytes. Whereas the in vivo studies did not directly show that the proximal NFAT1 sites was occupied, it was shown that EGR-1 was required for NFATc2-mediated CD40L expression via “allowing” NFATc2
transcriptional activity. This suggests that regulation of EGR-1 activity is an additional target for modulating platelet-mediated inflammatory activity. In addition to possible therapeutic significance, recent studies reveal that CD40L–dependent inflammatory activity of platelets is enhanced in several chronic inflammatory disease states, such as systemic lupus erythematosus and atherosclerosis, and that the enhanced inflammatory activity of platelets may contribute significantly to secondary pathology associated with progression of these diseases, including glomerulonephritis, plaque rupture, and increased thrombotic risk (4, 7, 44, 45). Recent studies have established a relationship between rheumatoid arthritis and pathologic thrombopoiesis via systemic inflammatory and synovial growth factors feeding back into the bone marrow (46, 47). Although it is not clear if the inflammatory signals feed back to the megakaryocyte compartment via NFAT/EGR-1 signaling, the possibility of megakaryocytes responding to systemic inflammation and dynamically altering platelet composition potentially expands the role of platelets beyond simply the ability to deliver early inflammatory signals to potential modulators of inflammatory disease progression and secondary pathology.

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