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*J Immunol* 2010; 185:6058-6067; Prepublished online 13 October 2010;
doi: 10.4049/jimmunol.1001682
http://www.jimmunol.org/content/185/10/6058

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/10/14/jimmunol.1001682.DC1

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PRDM1/Blimp-1 Controls Effector Cytokine Production in Human NK Cells

Matthew A. Smith,∗† Michelle Maurin,* Hyun Il Cho,* Brian Becknell,‡ Aharon G. Freud,‡ Jianhua Yu,‡ Sheng Wei,* Julie Djeu,* Esteban Celis,* Michael A. Caligiuri,‡ and Kenneth L. Wright∗∗†

NK cells are major effectors of the innate immune response through cytolysis and bridge to the adaptive immune response through cytokine release. The mediators of activation are well studied; however, little is known about the mechanisms that restrain activation. In this report, we demonstrate that the transcriptional repressor PRDM1 (also known as Blimp-1 or PRD1-BF1) is a critical negative regulator of NK function. Three distinct PRDM1 isoforms are selectively induced in the CD56dim NK population in response to activation. PRDM1 coordinately suppresses the release of IFN-γ, TNF-α, and TNF-β through direct binding to multiple conserved regulatory regions. Ablation of PRDM1 expression leads to enhanced production of IFN-γ and TNF-α but does not alter cytotoxicity, whereas overexpression blocks cytokine production. PRDM1 response elements are defined at the IFNG and TNF loci. Collectively, these data demonstrate a key role for PRDM1 in the negative regulation of NK activation and position PRDM1 as a common regulator of the adaptive and innate immune response.

The Journal of Immunology, 2010, 185: 6058–6067.

Natural killer cells play critical functions in innate and adaptive immunity. Although these lymphocytes were initially identified by their ability to lyse leukemia cells in a non-MHC–restricted manner, subsequent studies highlighted their role in cytokine production. In response to activating stimuli, NK cells proliferate, increase cytotoxicity, and produce cytokines, such as IFN-γ, TNF-α, and GM-CSF (1). IL-2 upregulates the expression of effector molecules and enhances natural cytotoxicity against a variety of targets. Furthermore, IL-2 and IL-15 signal through the common γR to control proliferation, with IL-15 being uniquely required for survival in vivo (2). IL-12 and IL-18 signal through distinct heterodimeric receptor complexes to elicit increases in IFN-γ via several mechanisms, including increased transcription, message stability, and nuclear retention (3–5). Synergistic increases in cytotoxicity and IFN-γ production are observed in response to costimulation with IL-12 and IL-18 (6, 7).

Cytokine-mediated activation of NK cells proceeds through several well-characterized nuclear transcription factors, many of which are functionally conserved between T and NK lineages (8). STAT4 is induced in response to IL-12 and is required for optimal IFN-γ production and increased cytotoxicity (9). IL-18 induces nuclear localization of NF-κB p50/p65 which, cooperatively with AP-1, increases IFN-γ and cytotoxicity (10). Furthermore, NFAT induces transcription of GM-CSF and TNF-α in NK cells (11). Conversely, relatively few negative regulators of activation-induced transcription have been identified in NK cells. ATF3 was recently shown to downregulate IFN-γ levels, and ATF−/− mice exhibit increased resistance to murine CMV infection (12). The transcription factor H2.0-like homeobox negatively regulates IFN-γ production, primarily through degradation of phosphorylated STAT4 not direct DNA-binding activity (13).

PRDM1 (also known as Blimp-1 or PRD1-BF1) is a transcriptional repressor encoded by the PRDM1 gene on chromosome 6q21. It was originally identified as a postinduction suppressor of IFNB in virally infected osteosarcoma cells (14). Subsequent work revealed a pivotal role in the terminal differentiation of Ab-producing plasma cells (15). We and other investigators previously showed that PRDM1 exerts its repressive functions through recruitment of histone-modifying enzymes (HDAC2, G9a, PRMT5, and LSD1) and Groucho corepressors (16–18). Through silencing of direct (cMyc, C/ETA, Pax5) and indirect targets, PRDM1 is a master regulator of terminal differentiation of B lymphocytes, mediating cell cycle exit, repression of early B cell factors, and induction of Ig secretion (19, 20).

More recently, a role for PRDM1 in T lymphocytes has emerged. PRDM1 is expressed in CD4 and CD8 T cell lineages and is critical for maintenance of homeostasis. Conditional knockout in T lymphocytes leads to increased effector populations, resulting in severe colitis (21, 22). Upon activation, an autoregulatory loop exists, whereby IL-2 induces PRDM1 expression, which, in turn, negatively regulates IL-2 transcription (23, 24). During CD4 polarization, PRDM1 is preferentially expressed in Th2 cells and reinforces commitment to this lineage through repression of Ifng, cfox, and tb21 (24, 25). Within the CD8 lineage, PRDM1 is expressed at higher levels in exhausted subsets and promotes acquisition of the effector phenotype through suppression of memory potential (26–28). Thus, in addition to well-characterized B cell-specific
functions, PRDM1 is a critical regulator of T lymphocytes. In this report, we provide a functional description of PRDM1 in NK cells.

Materials and Methods

Cells and cytokines

Primary human NK cells were isolated via negative selection using the EZeSep kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer’s instructions. Purity was verified by flow cytometry, and cells were routinely found to be 90–95% CD3 + CD8 - . Cells were maintained in RPMI 1640 (Life Technologies, Carlsbad, CA), supplemented with 10% FBS and 1% penicillin-streptomycin. For small in vitro experiments, cells were cultured in RPMI 1640 (Life Technologies, Carlsbad, CA), supplemented with 2% FBS. For stimulation, the following recombinant human cytokines were used: IL-2 (100 U/ml; PeproTech, Rocky Hill, NJ), IL-12 (10 ng/ml; PeproTech), IL-18 (100 ng/ml; MBL, Woburn, MA), TNF-α (20–100 ng/ml; eBioscience, San Diego, CA), and IFN-γ (10–50 ng/ml; eBioscience), α-IFN (10 or 50 ng/ml; Sigma-Aldrich, St. Louis, MO).

Mice

C57BL/6 mice (n = 4) were immunized i.v. with 250 μg polyinosinic-polycytidylic acid (poly-IC; Oncovir, Washington, DC) or PBS. Mice were sacrificed at 48 h postinjection, and single-cell suspensions were prepared from pooled spleocytes. Murine NK cells were isolated via negative selection using the Murine NK Enrichment Kit (StemCell Technologies). Lysates were prepared from purified NK cells and total spleocytes and analyzed by immunoblot.

Microarray hybridization and data analysis

Two micrograms of total RNA served as the mRNA source for microarray analysis. The poly(A) RNA was specifically converted to cDNA and then amplified and labeled with biotin, as described (29). Hybridization with the bidien-labeled RNA, staining, and scanning of the chips followed the prescribed procedure outlined in the Affymetrix technical manual. Scanned output files were visually inspected for hybridization artifacts and then prescribed procedure outlined in the Affymetrix technical manual. Heatmaps were generated with Heatmap Builder v1.1 using signal-intensity values of transcripts that were differentially expressed ≥2-fold. Transcripts with ≥3-fold increases/decreases are provided in Supplemental Table I, and complete data have been deposited at the Gene Expression Omnibus under accession number GE22919 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22919).

Western blotting

Whole-cell lysates from 5 × 10 6 cells were prepared in lysis buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 1% Nonidet P-40, 1% Na-deoxycholate, 0.1% SDS, 2 mM EDTA) on ice, sonicated, and separated by 8% SDS-PAGE. Gels were transferred to polyvinylidene difluoride, blocked with 5% skim milk, and probed using Abs directed against PRDM1 (PRDI-BF1) (1:1,000; Cell Signaling Technology), β-actin (1:10,000; Sigma-Aldrich). Secondary Abs conjugated to HRP were used for detection included anti-rabbit (1:2,000) and anti-mouse (1:10,000; both from GE, Pittsburgh, PA).

Real-time PCR

RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer’s instructions. Eluted RNA was treated with DNase and converted to cDNA, and one twentieth of the cDNA reaction was amplified and labeled with biotin, as described (29). Hybridization with the bidien-labeled RNA, staining, and scanning of the chips followed the prescribed procedure outlined in the Affymetrix technical manual. Scanned output files were visually inspected for hybridization artifacts and then prescribed procedure outlined in the Affymetrix technical manual. Heatmaps were generated with Heatmap Builder v1.1 using signal-intensity values of transcripts that were differentially expressed ≥2-fold. Transcripts with ≥3-fold increases/decreases are provided in Supplemental Table I, and complete data have been deposited at the Gene Expression Omnibus under accession number GE22919 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22919).

Adenoviral constructs and transduction

Adenoviral constructs were created in the Ad5/F35 vector from previously described constructs (16). This replication-deficient adenovirus uses the ubiquitously expressed CD46 molecule to mediate high-level transduction efficiency in a variety of hematopoietic cells (31). Briefly, PRDM1a in the AdTrack vector was recombined with Ad5/F35 to generate a bicistronic viral vector containing PRDM1a and GFP. Purified stocks were obtained by infecting Ad293T cells for 48 h and concentrating via CsCl banding, according to standard protocols. Viral titers were calculated using the QuickTiter Adenovirus Quantitation Kit (Cell Biolabs, San Diego, CA). For transduction experiments, Jurkat cells were transduced using a multiplicity of infection of 500 at a density of 1 × 10 6 cells/ml for 44 h before stimulation with PMA (1 μg/ml) and PHA (10 ng/ml) for 4 h.

DNA constructs and luciferase assay

A fragment of the human IFNG promoter (−507–120) was PCR cloned from human genomic DNA into pcR2.1. The fragment was then subcloned into pGL3-basic using the HindIII and Smal restriction sites to generate pGL3-IFNG WT, pGL3-IFNG mut was obtained via site-directed mutagenesis of the −254 site, changing residues AAAAGT to TCTAGA, which created a novel XbaI site (Mutagenex, Piscataway, NJ). pGL3-IFNG Δ was obtained by ligation of an XbaI-XbaI fragment obtained from pGL3- IFNG mut with the XbaI-NheI fragment of the pGL3-Basic vector. Transfections were performed using 20 μg total plasmid into 10 5 cells by electroporation at 250V, 1070 μF in 300 μl RPMI 1640 using a Bio-Rad Gene Pulser II. Cells were cultured for 36 h at 10 6 cells/ml. Cells were lysed in 500 μl Passive Lysis Buffer and assayed using the Dual Luciferase kit, per the manufacturer’s instructions (Promega, Madison, WI).

Statistical analyses

Two-tailed paired t tests were used for statistical analyses; p < 0.05 was considered significant. All calculations were performed in Microsoft Office Excel (Microsoft, Redmond, WA).

Results

Human NK cells alter expression of multiple effector molecules and transcription factors in response to cytokine stimulation

NK cells are well known for their ability to upregulate the production of effector cytokines and cytotoxic potential in response to IL-2 and other cytokines, such as IL-12, IL-15, and IL-18. However, the roles of sequence-specific, DNA-binding transcription factors in the modulation of NK activity are incompletely characterized. To directly address this, we isolated NK cells from peripheral blood of healthy donors and performed global gene-expression
profiling on RNA isolated immediately or after 24 h of stimulation in the presence of IL-2, IL-12, and IL-18 (Fig. 1). Purity of the NK population was determined by flow cytometry to be ≥96%, and this was confirmed by the absence of significant signals for transcripts associated with B cells, T cells, and monocytes in the microarray (Fig. 1B). Biological reproducibility was extremely close, giving an $R^2$ value of 0.967 and 0.970 between donors in freshly isolated and stimulated samples, respectively (Supplemental Fig. 1). In total, 541 genes were increased ≥3-fold in both donors, whereas 609 genes were decreased ≥3-fold following 24 h of stimulation (Fig. 1A, Supplemental Table I). As expected, genes encoding effector cytokines (e.g., IFNG, TNF, and CSF1) and numerous TNF family

![FIGURE 1. Human NK cells activate effector molecules and transcriptional regulators in response to cytokine stimulation. A. Heatmap depicting top 75 genes found to be up- or downregulated ≥3-fold in response to 24 h of stimulation with IL-2 (100 U/ml), IL-12 (10 ng/ml), and IL-18 (100 ng/ml) relative to time 0 h. Pixel density (highest values in each are black, lowest are white) represents average hybridization signal intensity from two donors after and before stimulation for increased and decreased genes, respectively. Fold changes are shown in parentheses. Presentation of expression data in this manner allows appreciation of the magnitude of expression and increases/decreases in response to stimulation. B. Average signal intensities of transcripts associated with non-NK cell lineages are shown from the 0-h samples to demonstrate the low frequency of contaminating cells. C. Heatmap depicting the top 15 up- and downregulated DNA-binding transcription factors as in A. D. Average signal intensities of selected secreted cytokines and TNF family members.](http://www.jimmunol.org/DownloadedFrom/)
members were found to be transcriptionally upregulated in response to stimulation (Fig. 1C). Decreased levels of the TGFβ1 transcript, which is constitutively expressed in NK cells, were also observed in response to stimulation, consistent with TGF-β1’s role as a negative regulator of activation/proliferation.

Of the top 150 transcripts found to be modulated, nearly 10% were sequence-specific DNA-binding transcription factors (Fig. 1D). Among these factors, the transcriptional repressor PRDM1 was increased in response to stimulation. PRDM1 has not been previously identified in NK cells; however, PRDM1 plays crucial roles in cell fate decisions and regulation of homeostasis within the immune system. The role of PRDM1 in the terminal differentiation of mature B cells into Ig-secreting CD138+ plasma cells is well established. More recently, roles for PRDM1 in the maintenance of homeostasis and effector versus memory lineage commitment in T lymphocytes were reported (26–28). This suggests that PRDM1 may also have key functional roles in NK cells.

**Cytokine stimulation induces multiple PRDM1 isoforms in human NK cells**

To directly establish PRDM1 activation in human NK cells, cells were isolated by negative selection and stimulated with multiple combinations of IL-2, IL-12, and IL-18. Immunoblot analysis revealed that freshly isolated and unstimulated NK cells have barely detectable levels of PRDM1 protein (Fig. 2A). Stimulation with IL-2 or a combination of IL-12 and IL-18 resulted in increased levels of PRDM1 protein, which is markedly enhanced by costimulation with all three cytokines. Cell lines derived from malignant NK tumors constitutively express PRDM1 and exhibit distinct isoform-expression patterns (Fig. 2B). YT and NK92 cell lines exclusively express the larger α isoform, whereas the NK cell line most closely resembles the expression pattern observed in primary human NKs.

Primary NK cells express three distinct molecular mass forms of PRDM1. The largest molecular mass band corresponds to the full-length PRDM1α isoform; however, this protein is significantly underrepresented compared with the smaller PRDM1 proteins. The predominant and smallest form corresponds to the PRDM1β isoform, which is characterized in multiple myeloma tumor cells and shown to have partially reduced repressive ability in reporter assays (32). In addition, immunoblot analysis detected an intermediate-sized isoform in primary NK cells. This protein migrating at ~85 kD likely represents the human homolog of a previously described murine splice variant prdm1Δexon7 (33). RT-PCR analysis using primers spanning the analogous exon in humans (exon 6) confirmed the presence of this splice variant (Supplemental Fig. 2). Consistent with our immunoblotting experiments, analysis of the mRNA levels specific for the α and β isoforms indicate that cytokine stimulation upregulates both isoforms and that PRDM1β mRNA is present at ~20-fold higher levels than is PRDM1α (Fig. 2C).

Human NK cells can be divided into two subsets based on the surface density of CD56 and the presence of CD16. The CD56bright CD16dimsubset represents ~10% of the human peripheral NK cell compartment and was suggested to play an immunomodulatory role based on its increased ability to produce cytokines; the CD56dimCD16 bright subset represents ~90% of human peripheral NK cells and is considered the primary cytotoxic subset. We questioned whether PRDM1 is restricted to specific subsets or whether it is broadly expressed in NK cells. Human NK cells were isolated from healthy donors via negative selection, followed by flow-cytometric sorting into CD56bright and CD56dim subsets. The cells were then stimulated with IL-12 and IL-18 for 24 h before analysis. Immunoblot and quantitative RT-PCR analysis revealed that PRDM1 is preferentially expressed in the CD56dim subset in response to stimulation (Fig. 2D, 2E). Furthermore, PRDM1β is the predominant isoform present.

**PRDM1 associates with NK cell activation**

Given that PRDM1 was maximally induced in response to combination stimulation with IL-2, IL-12, and IL-18, we hypothesized that levels of PRDM1 correlated with the degree of activation. We profiled mRNA levels of the effector cytokines IFN-γ and TNF-α in purified human NK cells after 24 h of stimulation with various combinations of cytokines. Quantitative RT-PCR analysis indicates that IFN-γ and TNF-α mRNA are synergistically increased upon stimulation with IL-2, IL-12, and IL-18 (Fig. 3A). Consistent with this, IL-2, IL-12, or IL-18 alone each minimally altered PRDM1
expression (Fig. 3B). Thus, PRDM1 levels correlate with effector cytokine transcription.

We next assessed whether effector cytokines induced PRDM1 through autocrine or paracrine feedback mechanisms. Treatment with IFN-γ or TNF-α failed to induce PRDM1 (Fig. 3C). Similarly, stimulation with α-IFN is insufficient to induce PRDM1, suggesting that induction results primarily from cytokine receptor-mediated signaling and likely requires multiple signaling events.

To determine whether PRDM1 is induced in vivo, we activated murine NK cells by poly-IC injections into C57BL6 mice. Poly-IC is known to activate NK cells indirectly through TLR3-mediated release of cytokines from dendritic cells and other accessory cells. NK cells were purified by negative selection from the spleens of naive and poly-IC–treated mice. PRDM1 is robustly induced specifically in NK cells upon 48 h of treatment (Fig. 4). PRDM1 expression was also detected in total splenocytes, and the level did not change with poly-IC treatment. This is likely due to the presence of PRDM1 in T cells and differentiating plasma cells within the spleen. Interestingly, only the PRDM1α homologous isoform was detectable in murine NK cells. Although an inability of the Ab to cross-react with other murine isoforms cannot be excluded, it is possible that the expression and/or function of PRDM1 isoforms in murine cells is differentially regulated.

**PRDM1 is not involved in perforin-mediated cytotoxicity**

The ability to lyse target cells in an Ag-independent manner is a hallmark of NK cells. Cytotoxicity against heterologous target cells proceeds through the release of perforin and granzymes and is markedly increased upon treatment with IL-2, IL-12, or IL-18 (1, 34). To assess whether PRDM1 regulates cytotoxicity, NK cells were isolated from healthy donors and stimulated with IL-2 and IL-12 or the combination of IL-2, IL-12, and IL-18 in the presence of a nontargeting (NT) control or a PRDM1-specific siRNA for 72 h. Cytotoxicity against the K562 leukemia cell line was assessed in a 4-h [51Cr]-release assay. As expected, cytokine-activated NK cells exhibit significant cytotoxicity against K562 targets (Fig. 5A). Knockdown of PRDM1 expression did not alter cytolytic activity across several E:T ratios. Distinct from our experiments that revealed increases in IFNG and PRDM1 mRNA levels with the addition of IL-18 to IL-2 and IL-12, we observed no additive effect of IL-18 in cytotoxicity assays, further demonstrating that cytotoxicity and PRDM1 levels are not directly linked. Knockdown of PRDM1 protein was confirmed by immunoblotting to be highly efficient, and it had no effect on viability, as assessed by trypan blue staining (Fig. 5B, data not shown). Furthermore, no activation-induced cell death was detected in stimulated NK cells, as measured by PARP cleavage (Fig. 5C). Collectively, these results show that PRDM1 does not have a significant role in regulating perforin-mediated cytotoxicity in NK cells.

**PRDM1 binds promoters in multiple target genes**

Because PRDM1 is well-documented as a DNA-binding transcriptional repressor, we sought to characterize its function in NK cells by identifying DNA elements to which PRDM1 specifically binds. To accomplish this, we performed chromatin immunoprecipitation (ChIP) experiments in primary NK cells isolated from healthy donors. Cells were stimulated for 24 h with IL-2, IL-12, and IL-18 to induce PRDM1 expression prior to isolation of chromatin and immunoprecipitation with anti-IgG or anti-PRDM1 Abs. Initially, we assayed promoter regions of genes known to be regulated by PRDM1 as measured by PARP cleavage (Fig. 5). Collectively, these results show that PRDM1 does not have a significant role in regulating perforin-mediated cytotoxicity in NK cells.

**FIGURE 4.** PRDM1 is induced in vivo. C57BL6 mice were treated with 100 μg poly-IC via tail vein injection for 48 h. NK cells purified via negative selection (left panel) or total splenocytes (right panel) were analyzed by immunoblot analysis.

**FIGURE 3.** PRDM1 expression associates with NK activation. A, RT-PCR analysis of cDNA synthesized from freshly isolated or NK cells stimulated for 24 h. Expression values were calculated using the ΔΔCt method with 18S as the control gene. Upon linearization of Ct values, average time 0 for each donor was arbitrarily set to “1”. Error bars represent SD from at least three biologically independent samples isolated from different donors at different times. B, Immunoblot analysis of purified human NK cells stimulated for 24 h with IL-2 (100 U/ml), IL-12 (10 ng/ml), and IL-18 (100 ng/ml) alone or in combination. C, Immunoblot analysis of lysates prepared from purified NK cells treated with recombinant human TNF-α (20 or 100 ng/ml), IFN-γ (10 or 50 ng/ml), α-IFN (10 or 50 ng/ml) or IL-2 (100 U/ml), IL-12 (10 ng/ml), and IL-18 (100 ng/ml) for 24 h.
cells. We observed PRDM1 binding at the IFN-γ-inducible CII-TapIV promoter but not the lymphoid-specific CIITA promoter. Although human NK cells can increase surface MHC class II expression in response to activation (35), they express low to undetectable levels of CIITA. Binding of PRDM1 to the CII-TapIV promoter may reinforce this repressed state and prevent IFN-γ-mediated autoinduction. We did not detect binding at the promoters of PAX5 and IFNB, both of which were shown to be directly regulated via binding of PRDM1 to promoters in B cells and osteosarcoma cell lines, respectively. Furthermore, we did not detect binding at the promoter of SLAMF7, which encodes the NK-activating receptor CD2-like receptor activating cytotoxic cell and contains a potential PRDM1-binding motif within its promoter. This indicates that PRDM1 binds target-gene promoters in a selective and cell type-specific manner. As expected, no binding was detected using primers to the second exon of myoglobin B, which was used as a negative control.

In murine T cells, Blimp-1 was recently shown to bind to a distal conserved regulatory site within the Ifng locus in vitro polarized CD4+ Th1 lymphocytes (25). Because NK cells are potent producers of IFN-γ during the early innate immune response, and induction is correlated with PRDM1 expression, we sought to evaluate PRDM1 binding to the human IFNG locus in cytokine-stimulated primary NK cells. We assayed four locations across the IFNG locus, which are highly conserved between rodents and humans and were demonstrated to regulate IFNG expression (36). We detected binding at the distal regulatory site, which has been demarcated as CNS −22 based on the genomic distances relative to the transcriptional start site in the mouse (Fig. 6C). This site is analogous to the PRDM1-binding site detected in T cells. Additionally, through bioinformatic analysis, we identified two potential PRDM1-binding sites within the minimal promoter located 370 and 254 bp upstream of the IFNG transcriptional start site. Very robust PRDM1 binding was detected in this proximal promoter region, which has not been identified in T cells. We did not detect binding at CNS −6 or 18–20, neither of which bears potential PRDM1-binding sites. Collectively, these data demonstrate that PRDM1 binds proximally and distally to multiple sites across the IFNG locus in human NK cells.

TNF is coordinately induced with IFNG upon cytokine stimulation of NK cells. Thus, we sought to determine whether this locus was also bound by PRDM1. To this end, we assayed four distinct locations across the ∼12-kb TNF locus by ChIP. The TNF locus contains three genes, each of which contains four exons (Fig. 6C). LTA and TNF are separated by ∼1 kb and are transcribed from the same strand, whereas LTB is transcribed from the opposite strand and is separated from TNF by ∼3 kb. We measured PRDM1 binding at the proximal promoter regions of LTB and TNF, the intergenic region between TNF and LTB, and a recently identified regulatory site located ∼3.5 kb upstream of LTA. This upstream enhancer element coordinately regulates the LTA and TNF genes but not the opposing LTB gene (37, 38). PRDM1 binding was clearly detected at this upstream regulatory site but not at either proximal promoter or the intergenic region. Furthermore, bioinformatic analysis detected a consensus PRDM1-recognition sequence in the bound enhancer element. These data indicate that PRDM1 specifically associates with defined regulatory sequences of the TNF locus.

Blockade of cytokine-induced PRDM1 expression increases effector cytokine production

Our experiments demonstrated that PRDM1 is coordinately induced with effector cytokines upon stimulation and occupies specific regulatory regions within the IFNG and TNF loci. To investigate the functional effects of PRDM1 in NK cells, we performed gene-expression knockdown (KD) experiments using pri-
mary human NK cells. Cells were stimulated with IL-2 and IL-12 in the presence of an NT control or PRDM1-specific siRNA. mRNA was isolated after 48 h and analyzed by real-time quantitative RT-PCR. We found significantly higher mRNA levels of IFNG, TNF, and LTA when PRDM1 induction was abrogated via siRNA, yet no significant differences were found for DAP10 (Fig. 7A). We next sought to determine whether these increases corresponded with detectable changes in protein expression. Secreted IFN-γ and TNF-α were measured by ELISA, and consistent increases in secreted protein were detectable in multiple donors in response to PRDM1 expression KD (Fig. 7B). Consistent with this silencing, PRDM1 expression remained elevated over a 4-d post-stimulation time course, whereas IFNG and TNF levels declined (Supplemental Fig. 3). Together, these data indicate that PRDM1 negatively regulates production of these cytokines in response to NK cell activation.

**Overexpression of PRDM1 mediates repression of activation-induced expression of IFNG and TNF**

Because we observed occupancy of PRDM1 at effector cytokine loci and increased production in the context of PRDM1 KD, we wanted to assess whether PRDM1 was capable of blocking activation-induced transcription of IFNG and TNF. The Jurkat T cell line was transduced with adenovirus expressing GFP alone or GFP and PRDM1α prior to stimulation. After PMA/PHA stimulation, quantitative RT-PCR analysis was performed to assess induction of IFNG and TNF. GFP-transduced cells showed significant upregulation of IFNG and TNF upon stimulation, which was nearly
abolished in PRDM1α-transduced cells (Fig. 8A). Thus, introduction of PRDM1 prior to stimulation was sufficient to block stimulation-induced transcription of IFNG and TNF, providing further support that PRDM1 is a negative regulator of effector cytokine production.

**PRDM1 mediates repression of IFNG via elements in the proximal promoter**

To directly assess the functionality of PRDM1 binding to the newly identified elements within the proximal promoter of the IFNG gene, we cloned the region containing −507 to +121 of the human IFNG gene and inserted it upstream of a luciferase reporter. This promoter region contains the two potential PRDM1 binding sites located at −370 and −254. Cotransfection of PRDM1 was sufficient to repress luciferase activity driven by the wild-type IFNG promoter by ~50% (Fig. 8B). To assess the relative contributions of the two potential PRDM1-binding motifs, a deletion of ~300 bp encompassing both of these sites (ΔIFNG-pGL3) was created.

This deletion eliminated ability of PRDM1 to repress transcription from the construct. This repression was similarly eliminated by point mutations at the −254 site (mutIFNG-pGL3), demonstrating that this location is critical for PRDM1-mediated repression. Collectively, these data demonstrate PRDM1-mediated repression of IFNG through binding to this previously uncharacterized motif.

**Discussion**

In this report, we provide the functional description of PRDM1 in NK cells. We showed that PRDM1 accumulates upon cytokine-mediated activation and acts as a negative regulator of activation, attenuating inflammatory cytokine production. Such a negative feedback loop has important implications in the context of inflammation and immune homeostasis.

Using global gene-expression profiling, we identified PRDM1 as a highly expressed transcription factor in stimulated NK cells. Our data complement previous studies (39–42), providing a global gene-expression profile of NK cells using a physiologically relevant combination of cytokines to achieve activation. During the early phases of the innate-immune response, IL-12 and IL-18 are produced by monocytes and epithelial cells, whereas IL-2 is primarily produced by activated T cells, typically a later event. Another early monocyte-derived cytokine, IL-15, signals through the shared IL-2R and can substitute for IL-2 to induce PRDM1 in the presence of IL-12 and IL-18 (M.A. Smith and K.L. Wright, unpublished observations). Consistent with this, maximal induction of murine NK cells in response to *Salmonella*-infected macrophages was recently shown to require IL-2 and/or IL-15, macrophage-derived IL-12 and IL-18, and direct NK cell–macrophage contact (6). Thus, the exposure of freshly isolated NK cells to these cytokines ex vivo can mimic the milieu present in vivo during the early innate-immune response and can provide insight into the physiological gene expression changes that are occurring.

The ability of NK cells to produce a variety of cytokines in response to stimulation provides a crucial mechanism of cross-regulation between the innate and adaptive arms of the immune system. To maintain homeostasis, regulatory control must be exerted upon the activation phase, as well as during the recovery phase to restrain the degree of activation. Although activation events have been thoroughly investigated, molecular events regulating dampening or recovery remain poorly characterized. This study now shows that PRDM1 is an important mediator of this phase. PRDM1 attenuates production of multiple effector cytokines in a coordinate manner via direct binding to specific DNA sequences in known regulatory regions. At the TNF locus, PRDM1 binds specifically to a highly conserved regulatory element ~3.5 kb upstream of the transcriptional start site of *LTA*. This region exhibits DNaese hypersensitivity and contains binding sites for inflammatory activators, such as NF-κB and NFAT (37). Furthermore, this site was shown to be crucial for activation-induced looping (38). This three-dimensional looped chromatin structure mediates interaction between distally and proximally bound NFAT at the TNF promoter, forming an enhanceosome resulting in transcriptional activation of TNF and, to a lesser extent, *LTA* in response to stimulation. Consistent with the notion that this region imparts specific localized control at the TNF locus, KD of PRDM1 results in increases in *LTA* and *TNF*, but not *LTB*, which is located further downstream and is encoded on the opposite DNA strand.

The IFNG locus is also subject to PRDM1-mediated regulation in NK cells. Studies in the T cell compartment identified an upstream regulatory region in murine CD4+ T cells (CNS-22) with enhancer activity that contributed to Tbet-dependent *Ifng* expression in vitro polarized Th1 cells (36). This site was later shown to be bound by the murine homolog Blimp1 (25). In our study, we...
confirmed the functionality of this distal site in human NK cells. In addition to this distal element, we identified a promoter-proximal element within the IFNG promoter to which PRDM1 binds. This proximal site is required for silencing of a reporter gene driven by the human IFNG promoter. Freshly isolated resting NK cells differ substantially from T cells, with respect to the kinetics of IFNG mRNA induction. Indeed, NK cells induce IFNG rapidly upon stimulation without the requirement for chromatin modifications, because the entire locus is poised in a hyperacetylated state under basal conditions (43). Thus, PRDM1 binding directly upstream of the transcriptional start site may facilitate potent silencing in NK cells, without necessarily requiring long-range chromatin modifications.

Human NK cells exist in at least two functionally divergent subsets based on the surface density of CD56 and CD16. Several groups demonstrated that CD56dimCD16+ NK cells are the major population in peripheral blood, whereas CD56brightCD16−/dim NK cells represent <10% of peripheral NK cells and primarily localize to lymph nodes (44). The CD56bright population has been described as a regulator population. These cells express the high-affinity IL-2R, are highly proliferative in response to stimuli, and localize at inflammatory sites. Accumulating evidence suggests that CD56bright cells may be developmental precursors to the more mature CD56dim effector population (45, 46). Conversely, the CD56dim effector population exhibits increased natural cytotoxicity, but it produces lower levels of inflammatory cytokines relative to the CD56bright population. Consistent with its role as a negative regulator of cytokine production, we observed that PRDM1 is preferentially expressed in the CD56dim population. Thus, PRDM1-mediated transcriptional repression likely contributes to the functional divergence observed in these populations.

NK cells exhibit a unique pattern of PRDM1 protein isoform expression. In addition to the full-length PRDM1α, NK cells express high levels of two smaller molecular mass species. Expression of the PRDM1β isoform was documented only in myeloma cell lines and samples from myeloma and T cell lymphoma patients, and it is always present at lower levels than is the full-length protein (32, 47). NK cells represent the first nontransformed cell type with PRDM1β expression; furthermore, it is consistently observed at higher levels than is the larger PRDM1α. The β isoform is transcribed from an alternate promoter and uses a distinct transcriptional start site present in the third intron of the full-length protein. The resulting protein has a disruption of the highly conserved PR domain. We previously showed that the β isoform localizes to the nucleus and maintains the capacity for DNA binding, although its repressive activity is dampened relative to PRDM1α. All of the cytokine combinations tested activated transcription of both isoforms in NK cells, although there remains the potential that isoform-specific activation signals may exist. We also consistently observed an intermediate-sized isoform (PRDM1αΔ6) corresponding to a deletion of the amino acids encoded by exon 6. This isoform is generated via splicing to exclude exon 6, resulting in deletion of the second zinc finger, as well as disruption of a portion of the first and third zinc fingers. An analogous splice variant was recently described in naïve CD19+ murine B cells, although it was present at very low levels (33). The first two zinc fingers of PRDM1 have a role in DNA binding and are required for recruitment of the histone methyltransferase G9a but are dispensable for interaction with HDAC2 (16, 48). Interestingly, we did not observe induction of apoptosis or evidence of cell cycle exit concomitant with PRDM1 induction in NK cells. Furthermore, IL-2–expanded LAK cells continued to proliferate, despite the acquisition of high-level PRDM1 expression (M.A. Smith and K.L. Wright, unpublished observations). Thus, the presence of these lower molecular mass isoforms that have altered or impaired activity might provide a cytoprotective effect, whereby NK cells are protected from the antiproliferative and apoptotic effect of PRDM1 but retain the ability to repress selective target genes. Characterization of the contribution of each isoform is an important area of future investigation.

The PRDM1 allele is encoded on chromosome 6 within a region that is known to be frequently associated with B, T, and NK cell-derived malignancies. A recent report used high-resolution comparative genomic-hybridization arrays to precisely map regions deleted within NK lymphomas and identified a minimal common region spanning ~2 Mb of the 6q21 deletion, which is present in ~40% of cases (49). Within this region, the investigators identified three genes that were downregulated in tumor specimens harboring the 6q21 deletion: ATG5, PRDM1, and AIM1. More recently, PRDM1-expression levels were shown to be highly variable and were found to be independent of the 6q21 deletion in an independent study of six primary NK lymphoma patient samples (50). Our data clearly establish a functional role for PRDM1 in normal NK cell function. Thus, although PRDM1 can act as a tumor suppressor in diffuse large B cell lymphoma, its contribution to the transformed phenotype in NK-derived malignancies remains controversial.

Our studies add NK cells to the expanding list of immune lineages in which PRDM1 has a key functional role. PRDM1 has been described in plasma cells, multiple T cell subsets, dendritic cells, and myeloid cells. Although lineage-specific regulation of target genes exists, a significant commonality is that PRDM1 regulates final effector function. For instance, negative regulation of Il2 and itox21 by Blimp1 promotes Th2 polarization in CD4+ T cell differentiation, yet the differential expression of these genes does not play a role in B or NK cell effector function. In contrast, coordinate regulation of IFNG and TNF is critical to T and NK cell effector function. Characterization of overlapping and nonoverlapping functions of PRDM1 across multiple lineages will be an important area of focus in future experiments. For example, Lanier and colleagues (51) recently provided evidence for a memory phenotype in murine NK cells using a murine CMV infection model wherein they demonstrated that Ag-specific Ly49H+ NK cells underwent a prolonged contraction period but were subsequently activated to higher levels upon restimulation. PRDM1-mediated restriction of memory potential was recently described in CD8+ T cells (26). Given that PRDM1 is induced in vivo during NK activation, it will be interesting to ascertain whether it has such a role in NK memory commitment.

In summary, our data suggest that PRDM1 plays a crucial role in the postactivation phenotype of NK cells by negatively regulating cytokine transcription in a coordinate manner, without compromising perforin-mediated cytotoxicity or inducing exit from the cell cycle. Such a mechanism may have important implications in innate immunity and tumor surveillance.

Acknowledgments
We thank the staff of the Microarray and Flow Cytometry Core Facilities at H. Lee Moffitt Cancer Center. We also thank Xiaolong Fang for providing recombinant adenoviral vectors and Jerome Ritz (Dana-Farber Cancer Institute, Boston, MA) for providing the NKL cell line.

Disclosures
The authors have no financial conflicts of interest.

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