

The PP2A inhibitor SET regulates natural killer cell IFN- γ production

Rossana Trotta,¹ David Ciarlariello,¹ Jessica Dal Col,¹ Jeffrey Allard II,¹ Paolo Neviani,¹ Ramasamy Santhanam,¹ Hsiaoyn Mao,¹ Brian Becknell,^{1,2} Jianhua Yu,¹ Amy K. Ferketich,³ Brittany Thomas,¹ Aalok Modi,¹ Bradley W. Blaser,^{1,2} Danilo Perrotti,^{1,5} and Michael A. Caligiuri^{1,4,5}

¹Department of Molecular Virology, Immunology and Medical Genetics, ²Medical Scientific Program, Integrated Biomedical Graduate Program, College of Medicine and Public Health; ³Division of Epidemiology, College of Public Health; ⁴Division of Hematology and Oncology, Department of Internal Medicine; and ⁵The Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

Monokines (i.e., interleukin [IL]-12, -18, and -15) induce natural killer (NK) cells to produce interferon- γ (IFN- γ), which is a critical factor for immune surveillance of cancer and monocyte clearance of infection. We show that SET, which is a potent inhibitor of protein phosphatase type 2A (PP2A) activity, is highly expressed in human CD56^{bright} NK cells, which produce more IFN- γ than CD56^{dim} NK cells. SET was up-regulated upon monokine stimulation of primary human NK cells. Furthermore, ectopic overexpression of SET significantly enhanced IFN- γ gene expression in monokine-stimulated NK cells. In contrast, RNAi-mediated suppression of SET expression renders NK cells inefficient in producing high levels of IFN- γ in response to monokine costimulation. Mechanistically, suppression of PP2A activity by SET is important for IFN- γ gene expression in NK cells. In fact, treatment of primary human NK cells with the PP2A activator 1,9-dideoxy-forskolin, as well as administration of the drug to C57BL/6 mice, significantly reduced NK-dependent IFN- γ production in response to monokine treatment. Further, SET knockdown or pharmacologic activation of PP2A diminished extracellular signal-regulated kinase 1/2, p65RelA, signal transducer and activator of transduction 4 (STAT4), and STAT5 activity in monokine-stimulated NK cells, potentially contributing to the reduction in IFN- γ gene expression. Thus, SET expression is essential for suppressing PP2A phosphatase activity that would otherwise limit NK cell antitumoral and/or antiinflammatory functions by impairing NK cell production of IFN- γ .

CORRESPONDENCE

Rossana Trotta:
rossana.trotta@osumc.edu;
OR
Michael A. Caligiuri:
michael.caligiuri@osumc.edu

Abbreviations used: APC, allophycocyanin; CTS, cytotstatin; ERK, extracellular signal-regulated kinase; I1PP2A, PP2A inhibitor 1; I2PP2A, PP2A inhibitor 2; MAP, mitogen-activated protein; MAPK, MAP kinase; PP2A, protein phosphatase type 2A; shRNA, short hairpin RNA; PP2Ac, PP2A catalytic subunit; STAT, signal transducer and activator of transduction.

Human NK cells are CD56⁺CD3⁻ large granular lymphocytes of the innate immune system (1). NK cells participate in early responses against infection or malignant transformation through production of cytokines and chemokines and via direct cytotoxicity in the absence of cognate MHC ligands (2, 3). Additionally, NK cells regulate the adaptive immune response by interacting with DCs and by polarizing CD4⁺ helper T cell cytokine production toward a Th1 response (4–6). Two functionally distinct subsets of human NK cells can be identified by their surface expression of CD56 (7). CD56^{dim} NK cells are more abundant in blood than CD56^{bright} cells, whereas the opposite is true in LNs (8). Functionally, CD56^{dim}

NK cells are efficient effectors of natural and antibody-dependent cytotoxicity, whereas the CD56^{bright} NK cells produce abundant immunoregulatory cytokines and chemokines in response to monokine costimulation (9, 10).

Monokine-activated NK cells produce proinflammatory cytokines required for monocytes/macrophage clearance of pathogens and effective tumor surveillance (11–14). The prototypic cytokine is IFN- γ , and it is produced by NK cells in response to IL-1, -12, -15, and -18, cytokines for which NK cells express functional receptors (3, 9). NK IFN- γ in turn promotes the maturation and activation of monocytes, and is a potent activator of macrophage functions such as antigen presentation and phagocytosis (15). The activation of NK cell IFN- γ production is a complex process involving a

M.A. Caligiuri and D. Perrotti contributed equally to this paper.
The online version of this article contains supplemental material.

multitude of positive and negative mediators, such as kinases and phosphatases, as well as transcription factors (16–18).

The SET (I2PP2A, IGAAD, and TAF1b) protein is localized to the nucleus and cytoplasm and has a critical role in the regulation of normal and cancer signal transduction (19). In fact, SET is a potent inhibitor of protein phosphatase 2A (PP2A) activity (20, 21), even though it has also been described as an inhibitor of the tumor suppressor NM23-H1, which is a granzyme A DNase-activated factor (22), and a negative regulator of histone acetylation (23). PP2A is the major cellular serine threonine phosphatase involved in the regulation of a variety of cellular processes and signal transduction pathways (19, 24, 25), yet its role in the regulation of NK cell biology has not been fully explored. There is some evidence that PP2A may be involved in the expansion and activation of mouse NK cells *in vivo*. In fact, the *in vivo* administration of the PP2A inhibitor cytostatin (CTS) can inhibit B16 melanoma pulmonary metastasis, possibly via the up-regulation of cytokines that are important for NK cell development and/or activation (26).

We show that human NK cell subsets differentially express SET and use it to regulate NK IFN- γ gene expression. The involvement of SET in the regulation of NK IFN- γ production is strictly dependent on its ability to suppress PP2A activity. This also prevents the PP2A-mediated dephosphorylation of extracellular signal-regulated kinase (ERK), p65RelA, signal transducer and activator of transduction (STAT) 4, and STAT5, which are signaling intermediates known to be involved in monokine-activated NK cell induction of IFN- γ (27–34).

RESULTS

SET expression in resting and monokine-activated human NK cells

Stimulation of NK cells with IL-12 and -18 induces a rapid increase in the expression of IFN- γ (3, 7, 9). Using a gene chip screen to assess potential regulatory mechanisms of IFN- γ production in NK cells, we found SET mRNA significantly up-regulated upon costimulation of NK-92 cells (a human CD56^{bright} NK cell line) (35) with IL-12 and -18 (unpublished data). Accordingly, up-regulation of SET mRNA (Fig. 1 A, left) and protein (Fig. 1 A, right) was observed within 6 and 8 h of monokine stimulation of NK-92 cells. Likewise, overnight costimulation of primary human NK cells with IL-12/-18- or IL-12/-15-enhanced SET mRNA (Fig. 1 B, left) and protein (Fig. 1 B, right) expression. Similar effects on induction of SET expression were noted after single-monokine (i.e., IL-12, -15, or -18) stimulation (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070419/DC1>). The IL-12/-18-mediated up-regulation of SET was not altered by neutralizing secreted IFN- γ (Fig. S2). Interestingly, the resting CD56^{bright} NK cell subset, which has a significantly enhanced capacity to invoke a cytokine response to monokine costimulation, constitutively expresses relatively high levels of SET protein compared with the more cytolytic CD56^{dim} NK cell subset (Fig. 1 C).

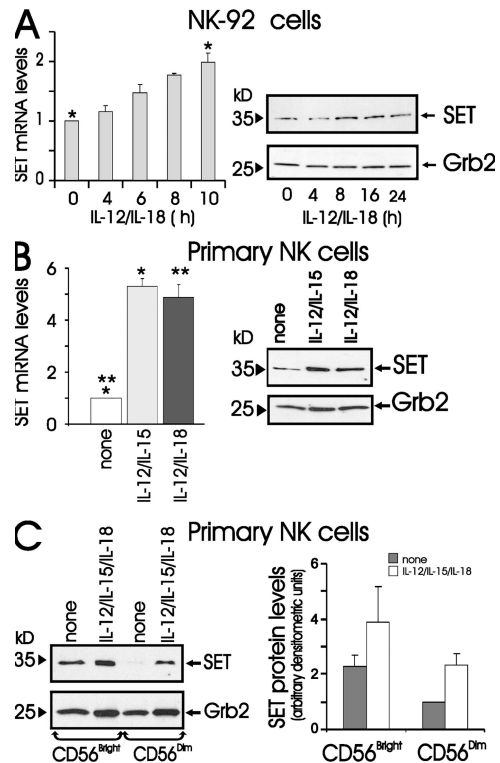


Figure 1. SET expression in NK cells. (A) NK-92 cells were costimulated with the monokines IL-12 (10 ng/ml) and -18 (100 ng/ml) for the indicated times, after which cell pellets were collected and analyzed for SET transcript by real-time RT-PCR (*, $P < 0.03$; $n = 5$; left) and for protein by Western blot (right). Human CD56⁺CD3⁻ primary NK cells (B) and CD56^{bright} and CD56^{dim} primary NK subsets (C) were either unstimulated (none) or costimulated with the monokines IL-12 (10 ng/ml) and -18 (100 ng/ml) and/or -15 (100 ng/ml) for 14 h. Cells were analyzed for SET transcript levels by real-time RT-PCR (* and **, $P < 0.03$; $n = 6$, both conditions; B, left) and for SET protein levels by Western blot (B [right] and C). Western blots were performed with anti-SET and -Grb2 antibodies sequentially on the same filter. Western blot results are representative of no less than three such experiments with virtually identical results. The densitometry analysis of SET protein levels in resting and monokine-activated CD56^{bright} and CD56^{dim} NK cells are illustrated in C (right), and the graph summarizes the data from three different experiments. Error bars in A and B represent the SEM of triplicates in one representative experiment, and in C the represent the SEM of triplicates in three experiments.

Role of SET in the regulation of IFN- γ production by monokine-stimulated NK cells

Based on the observation that IL-12, -15, and -18 monokines increase SET levels and that CD56^{bright} NK cells express more SET protein than CD56^{dim} NK cells, we hypothesized that SET could be a positive regulator for IFN- γ production in monokine-costimulated NK cells. Thus, we interfered with SET expression by (a) retrovirally infecting the NK-92 cells with pSUPER.retro-GFP/neo vector (pSUPER) carrying a short hairpin RNA (shRNA) corresponding to nucleotide 120–138 of human SET and GFP (pSUPER.retro-shSET) (21); and (b) ectopically expressing an Epstein-Barr virus/retroviral PINCO hybrid vector containing SET and GFP

cDNAs (PINCO-SET) in NK-92 and primary human NK cells. In all experiments using these infected cells, sorting was first performed to enrich for the GFP⁺ fraction.

As expected, expression of the SET shRNA in the GFP⁺ NK-92 cell fraction down-regulated SET expression at mRNA (not depicted) and protein levels (Fig. 2 A, inset) without affecting the expression of pp32 (PP2A inhibitor 1 [I1PP2A]) or that of the PP2A catalytic (PP2Ac) subunit (Fig. 2 A, inset).

Importantly, down-regulation of SET led to a marked reduction in IFN- γ mRNA expression (Fig. 2 A) and IFN- γ protein (Fig. 2 B) by NK-92 cells in response to costimulation with IL-12/-18, IL-15/-18, and, to a lesser extent, IL-12/-15. In contrast, IFN- γ production was enhanced in GFP⁺ PINCO-SET-transduced NK-92 cells compared with

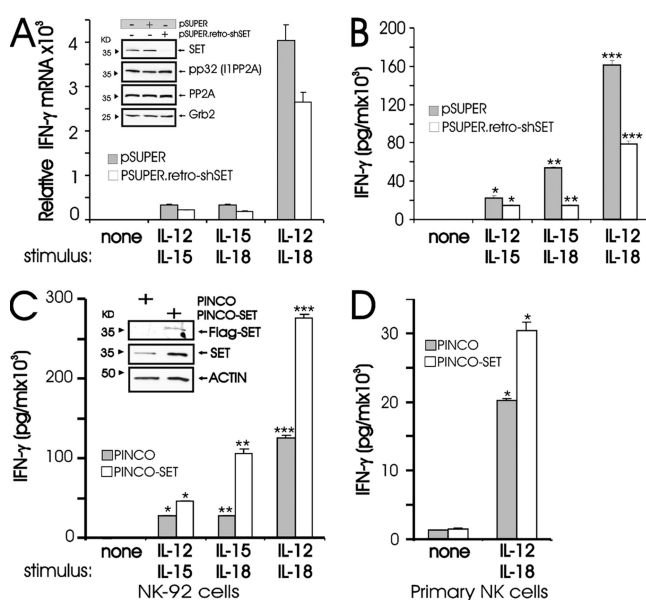


Figure 2. Effect of SET downmodulation and overexpression on IFN- γ production in monokine-stimulated NK cells. (A and B) NK-92 cells were retrovirally infected using the pSUPER or pSUPER.retro-shSET vector. Infected cells were FACS sorted for GFP and grown in the presence of 0.5 mg/ml G418. SET protein levels (A, inset) were analyzed by Western blot. Protein lysates were blotted with anti-SET, -I1PP2A, -PP2Ac, and -Grb2 antibodies. This experiment is representative of four experiments performed with similar results. pSUPER- and pSUPER.retro-shSET-infected NK-92 cells were enriched by sorting for GFP⁺ cells, stimulated for 18 h with various combinations of IL-12 (10 ng/ml), -18 (20 ng/ml), or -15 (100 ng/ml), and then quantified for induction of IFN- γ transcript by real-time RT-PCR (left) or secretion of IFN- γ protein by ELISA (*, **, and ***, $P < 0.004$; $n = 6$, for all conditions, ELISA; right). This experiment is representative of six performed with similar results. Similar significant ($P < 0.009$ for all conditions) results were obtained after only 4 h of monokine activation (not depicted). NK-92 cells (C) and primary human NK cells (D) were retrovirally infected using the PINCO or PINCO-SET vector. Infected cells were FACS sorted for GFP. SET protein levels were analyzed by Western blot (C, inset). Protein lysates were blotted with anti-SET, -Flag, and -actin antibodies. GFP⁺ PINCO- and PINCO-SET-infected NK-92 (C) and primary human NK cells (D) were stimulated for 18 h with IL-12, -18, and/or -15 and quantified for secretion of IFN- γ protein by ELISA (C: *, **, and ***, $P < 0.04$, $n = 4$, for all conditions; D: *, $P < 0.002$, $n = 4$). Error bars represent the SEM of triplicates in one representative experiment.

PINCO-transduced NK-92 cells, which were both costimulated with the aforementioned monokine combinations (Fig. 2 C). Similarly, a significantly higher amount of IFN- γ was detected in IL-12/-18-stimulated CD56⁺GFP⁺-sorted primary human NK cells infected with PINCO-SET compared with PINCO-transduced cells (Fig. 2 D).

SET up-regulates IFN- γ gene expression through suppression of PP2A activity

To determine whether the monokine-induced and SET-mediated induction of IFN- γ depend on the ability of SET to suppress PP2A activity in NK cells, PP2Ac was immunoprecipitated from lysates of pSUPER.retro-shSET- and pSUPER-transduced NK-92 cells, and immunoprecipitates

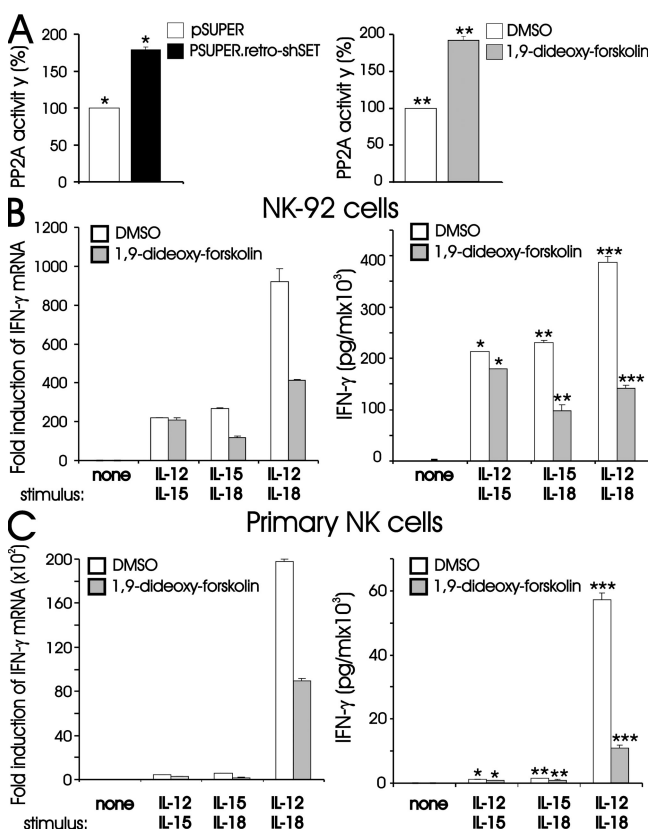


Figure 3. Pharmacologic induction of PP2A activity reduces IFN- γ gene expression in monokine-activated NK cells. (A) PP2A phosphatase assay was performed using PP2A immunoprecipitates obtained from cellular extracts of pSUPER- and pSUPER.retro-shSET-infected NK-92 cells (*, $P < 0.001$; $n = 4$; left) or primary NK cells treated with the PP2A activator 1,9-dideoxy-forskolin (**, $P < 0.001$; $n = 4$; right). NK-92 (B) and primary human NK cells (C) were first incubated (6 or 18 h) with medium containing either DMSO vehicle control or 1,9-dideoxy-forskolin, followed by an additional 18 h of incubation in the presence of IL-12 (10 ng/ml), -18 (100 ng/ml), and/or -15 (100 ng/ml). Cell pellets were then collected for quantification of IFN- γ transcript by real-time RT-PCR (B and C, left) and supernatants were collected and quantified for IFN- γ protein production by ELISA (B and C: *, **, and ***, $P < 0.008$, $n = 5$, for all conditions as quantified by ELISA; right). This is representative of four experiments performed with similar results. Error bars represent the SEM of triplicates in one representative experiment.

were used in phosphatase assays with PP2A-suitable phosphopeptide as substrate (21). Downmodulation of SET significantly augmented PP2A catalytic activity in NK-92 cells (Fig. 3 A, left). Additionally, treatment with 1,9-dideoxy-forskolin, which is a forskolin derivative that lacks adenylate cyclase-activating function while retaining the ability to activate PP2A (21, 36), efficiently induced PP2A activity in primary human NK cells (Fig. 3 A, right) and NK-92 cells (not depicted). Furthermore, PP2A activation by 1,9-dideoxy-forskolin (Fig. 3, B and C) and forskolin (not depicted) resulted in decreased IFN- γ mRNA (left) and secreted protein levels (right) in monokine-activated NK-92 (Fig. 3 B) and primary NK (Fig. 3 C) cells. PP2A activation by 1,9-dideoxy-forskolin inhibited monokine-induced IFN- γ production also in NK-92 cells ectopically overexpressing SET (Fig. 4, left).

To further strengthen the functional relationship between the SET-PP2A interplay and IFN- γ production, we pre-treated (1 h) SET shRNA-expressing (pSUPER.retro-shSET) and pSUPER-transduced NK-92 cells with the serine/threonine phosphatase inhibitor okadaic acid (Fig. 4, middle) and the specific PP2A inhibitor CTS (Fig. 4, right) (37) and used them to assess changes in the levels of IFN- γ secreted by IL-12/-18-costimulated NK-92 cells. As expected, both okadaic acid and CTS induced IFN- γ production in pSUPER.retro-shSET-transduced cells (Fig. 4, lane 2, middle and right), and also rescued IFN- γ production, albeit not completely, in pSUPER.retro-shSET-expressing cells (Fig. 4, lane 4, middle and right).

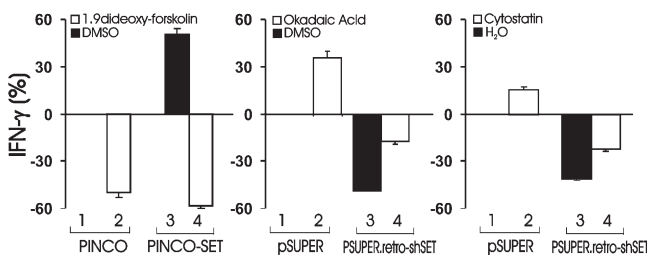


Figure 4. Modulation of PP2A overrides SET-mediated regulation of IFN- γ production in human NK cells. PINCO and PINCO-SET NK-92 cells were first incubated for 24 h at 37°C in medium containing DMSO vehicle (1 and 3) or 40 μ M of 1,9-dideoxy-forskolin (2 and 4), and then stimulated overnight in the presence of IL-12 plus -18. pSUPER- and pSUPER.retro-shSET-infected NK-92 cells were treated for 1 h with vehicle (DMSO or water; 1 and 3), 20 nM of the PP2A inhibitor okadaic acid (2 and 4), or 2 μ g/ml of the PP2A inhibitor CTS (2 and 4), followed by overnight incubation in IL-12 plus -18. Supernatants were collected and assayed for IFN- γ protein by ELISA. Results are representative of three experiments, and expressed as a percentage of IFN- γ increase or decrease compared with PINCO- or pSUPER-infected NK-92 cells stimulated with IL-12 plus -18 in a fashion identical to experimental wells. The absolute value of IFN- γ for 0% of the monokine-activated, DMSO-treated, PINCO-infected NK-92 cells is 61,541 pg/ml (left); the absolute value of IFN- γ for 0% of the monokine-activated, DMSO-treated, pSUPER-infected NK-92 cells is 430,854 pg/ml (middle); and the absolute value of IFN- γ for 0% of the monokine-activated, H₂O-treated, pSUPER-infected NK-92 cells is 124,456 pg/ml (right). Error bars represent the SEM of triplicates in one representative experiment.

To further demonstrate that SET-dependent suppression of PP2A activity is a necessary step for the induction of IFN- γ by monokine-stimulated NK cells, 1,9-dideoxy-forskolin (8 mg/kg/day; LD₅₀ = 68 mg/kg) or vehicle (2% DMSO) was administered i.p. for 3 consecutive days to C57BL/6 mice (n = 5 mice per group). To stimulate in vivo IFN- γ production by NK cells, IL-12 (1 μ g/mouse) and -18 (1 μ g/mouse) were co-administered i.p. to all mice 5 h after the last injection with vehicle or 1,9-dideoxy-forskolin. 18 h after monokine stimulation, vehicle- (DMSO) and 1,9-dideoxyforskolin-treated mice were killed, and intracellular staining for IFN- γ was performed on splenocytes labeled with anti-NK1.1 and CD3 antibody. Consistent with the existence of a functional link between SET expression, PP2A activity, and IFN- γ production in NK cells, gated NK1.1⁺CD3⁻ NK cells from 1,9-dideoxy-forskolin-treated mice produce significantly less IFN-g than NK cells from DMSO-treated mice (Fig. 5 B). Similar to the ex vivo experiments with human NK cells, the in vivo administration of IL-12 and -18 also induced substantially less IFN- γ transcript in splenocytes obtained from 1,9-dideoxy-forskolin-treated mice compared with vehicle-treated mice (Fig. 5 A). Thus, the ability of SET to regulate IFN- γ gene expression is dependent on its inhibitory control of PP2A activity.

Evidence for SET as a regulator of additional NK cell functions

We conducted some additional preliminary experiments to determine if SET might have a role in other NK cell functions. SET regulation of chemokine MIP-1 α gene expression is similar to that seen with IFN- γ , yet more modest in its effect (unpublished data). Down-regulation of SET in NK-92 cells infected with pSUPER.retro-shSET markedly diminishes their proliferative capacity compared with NK-92 cells infected with the pSUPER control (unpublished data). Finally,

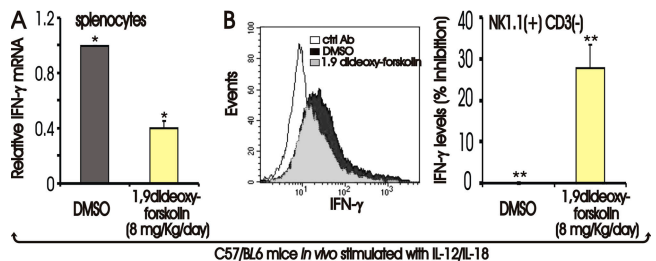


Figure 5. Effect of PP2A activation on IFN- γ production in vivo. C57BL/6J mice, treated for 3 d with vehicle (DMSO in PBS; 5 mice) or 1,9-dideoxy-forskolin (8 mg/kg/d; 5 mice), received an IP injection of 1 μ g IL-12 plus 1 μ g IL-18. After 18 h, spleens were harvested and splenocytes were collected for immediate quantification of IFN- γ transcript (*, P < 0.001, n = 5; A) or cultured for 4 h in Brefeldin A, stained with NK1.1-APC and CD3-FITC antibodies, permeabilized, and stained with an anti-mouse IFN- γ -PE or control isotype-PE mAb (B). The histogram (middle) and graph (right) show the results of flow analysis for IFN- γ in NK1.1⁺CD3⁻ NK cells from DMSO or 1,9-dideoxy-forskolin-treated mice as IFN- γ inhibition (mean percentage; **, P < 0.001; n = 5). Error bars represent the SEM of data obtained from five mice.

down-regulation of SET diminishes NK cytotoxicity (Fig. S3 A, available at <http://www.jem.org/cgi/content/full/jem.20070419/DC1>), whereas overexpression of SET promotes NK cytotoxicity (Fig. S3 B). In addition, the activator of PP2A, 1,9-dideoxy-forskolin, inhibits NK cell cytotoxicity in NK-92 cells that are overexpressing SET (Fig. S3 B).

Downstream signaling events modulated by the SET-PP2A interplay

Because PP2A targets several signaling molecules that reportedly influence IFN- γ gene expression, we investigated whether the activity of some of these positive regulators of NK IFN- γ expression (e.g., ERK, NF- κ B, STAT4, and STAT5) (27–34) was altered in NK cells upon molecular or pharmacologic modulation of the SET-PP2A interplay. Western blot analyses revealed that downmodulation of the PP2A inhibitor SET by shRNA or activation of PP2A by 1,9-dideoxy-forskolin treatment resulted in decreased levels of phosphorylated ERK^{Thr202/Tyr204}, STAT5^{Tyr694}, STAT4^{Tyr693}, and NF- κ B p65^{Ser536} in monokine-stimulated NK-92 cells (Fig. 6, A and B). In contrast, levels of phosphorylated p38^{Thr180/Tyr182} in monokine-stimulated NK-92 cells was not diminished in the presence of 1,9-dideoxy-forskolin (unpublished data), suggesting that this effect is not global for all signaling intermediates involved in these monokine activation pathways. As expected, CD56^{bright} primary human NK cells that constitutively express

high levels of SET (Fig. 1 C) have diminished PP2A activity (Fig. 6 C, left) and increased ERK phosphorylation (Fig. 6 C, right). In contrast, low SET expression in CD56^{dim} NK cells (Fig. 1 C) correlated with increased PP2A and decreased ERK activity (Fig. 6 C, left and right).

DISCUSSION

NK cells are important components of the innate immune system not only because of their ability to mediate cytotoxicity against pathogen-infected or tumor cells but also for their ability to produce immunoregulatory molecules, such as chemokines and cytokines (3). Specifically, NK cells produce IFN- γ , whose fundamental role during inflammation and tumor immunity has been well established. IFN- γ deficiency results in increased susceptibility to infection and/or malignancy (14, 38), whereas overproduction of IFN- γ leads to autoimmune disorders (39). Hence, a complete understanding of the molecular pathways that regulate IFN- γ expression is of fundamental importance, and the discovery of new regulators can help to identify new therapeutic targets for chronic inflammation and/or cancer.

In this study, we identified SET as a novel positive regulator of IFN- γ production by NK cells. In particular, we discovered that SET protein is constitutively abundant in resting CD56^{bright} NK cells, which are the subpopulation of NK cells that produce higher amounts of IFN- γ , compared with CD56^{dim} NK cells (7). We found that potent proinflammatory monokines (IL-12, -15, and -18) that stimulate NK cell IFN- γ production in NK cells via the inhibition of PP2A serine/threonine phosphatase activity and, further, that down-regulation of SET or enhanced PP2A activity decrease activation of signaling molecules associated with the induction of IFN- γ gene expression. Collectively, these data establish that SET expression is involved in the regulation of IFN- γ expression in human NK cells. Notably, to our knowledge, this represents the first investigation of the SET-PP2A axis in the human immune response. Future studies may extend the role of SET to other cell types and functions of the immune system.

Up-regulation of SET mRNA expression in monokine-activated primary NK cells directly correlates with the dynamics of SET protein expression, suggesting that induction and/or stabilization of the SET transcript and protein are tightly linked in this instance. The difference in constitutive SET expression found in resting CD56^{bright} and CD56^{dim} NK subsets may depend, at least in part, on their distinct micro-environments. CD56^{bright} NK cells develop in the T cell- and DC-rich parafollicular region of human LNs, where DC-derived IL-12, -15, and -18 may promote higher SET expression in this population (8). In contrast, highly cytolytic CD56^{dim} NK cells predominate in blood where monokine levels and cell-cell contact are negligible, such that SET expression is lower in this population. Alternatively, it is conceivable that CD56^{dim} NK cells uniquely possess an intrinsic signaling program that actively suppresses SET expression.

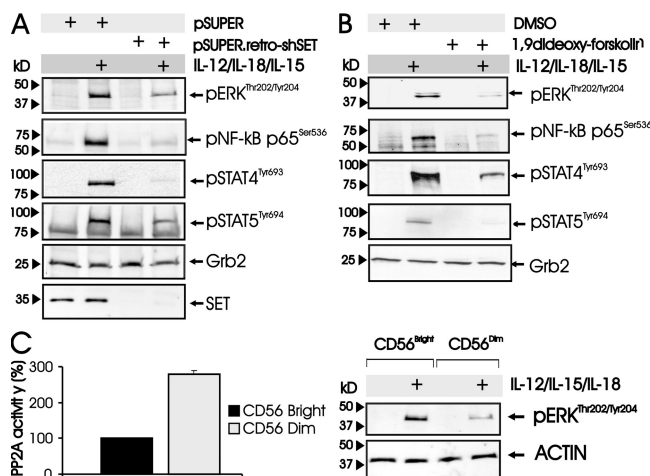


Figure 6. Reduction of SET or induction of PP2A results in a diminution of proinflammatory signaling intermediates involved in NK cell IFN- γ gene expression. Effect of ectopic SET shRNA expression (and consequent down-regulation of SET; A) or pharmacological activation of PP2A (B) on the levels of phospho-STAT5^{Tyr694}, -STAT4^{Tyr693}, -ERK^{Thr202/Tyr204}, and -NF- κ B p65^{Ser536} after costimulation of NK-92 cells with 10 ng/ml IL-12, 100 ng/ml IL-15, and 100 ng/ml IL-18 for 5 min. (C) PP2A phosphatase assay was performed using PP2A immunoprecipitates obtained from cellular extracts of unstimulated CD56^{bright} and CD56^{dim} primary NK subsets (left). CD56^{bright} and CD56^{dim} NK cells were either unstimulated or costimulated with monokines for 5 min, and immunoblotting for phospho-ERK^{Thr202/Tyr204} and actin was performed on the same filter (right). The illustrated data are representative of no less than three such experiments with identical results. Error bars represent the SEM of triplicates in one representative experiment.

The regulation of IFN- γ production by human NK cells, as with any process in any cell type, is achieved through the integration of dynamic positive and negative signals that reflect the intrinsic properties of the cell and its external milieu. Whereas monokines synergistically induce IFN- γ transcription, the immunoregulatory TGF- β attenuates IFN- γ transcription (18). Additionally, NK cells achieve negative feedback regulation of IFN- γ production through the monokine-inducible expression of the transcription factor Hlx (40). In CD56^{bright} NK cells, the monokine-induced IFN- γ production is also partially dependent on the expression and activity of SHIP1 inositol phosphatase. As SHIP1 is responsible for inactivation of AKT, ERK, and p38 mitogen-activated protein kinase (MAPK) pathways, it is intuitive that both resting and activated CD56^{bright} NK cells exhibit very low levels of SHIP1 (17). Interestingly, SET mRNA and protein levels are lower in NK cells derived from SHIP1-null mice than NK cells from wild-type animals (unpublished data). However, whether there is a direct link between SHIP1 and SET expression in human NK cells remains to be investigated.

Data from the current study suggest a potential model whereby monokines not only directly activate signaling molecules such as MAP kinases, NF- κ B, and JAK/STATs, which induce IFN- γ gene expression in NK cells, but also work via the induction of SET to inhibit the activity of PP2A, which is a potent and negative regulator of these pathways (Fig. 7). PP2A is a heterotrimeric enzyme that consists of a scaffold (A) subunit, a catalytic (C) subunit, and one of a diverse array of regulatory (B) subunits (19). The extensive number of different regulatory subunits enables PP2A to act on a wide range of signaling substrates (24). In particular, PP2A has been shown to modulate the activities of kinases or their substrates, such as ERK MAPKs, JAKs, JNK, PKB, IKK kinases, and Rel A, resulting in the regulation of numerous transcriptional factors, such as AP-1, NF- κ B, and STATs (21, 24, 41–43).

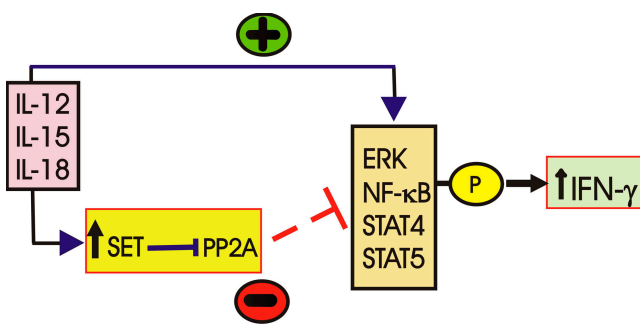


Figure 7. A model for the role of SET in regulating IFN- γ gene expression of NK cells after monokine costimulation. A variety of proinflammatory monokines can induce phosphorylation of positive signaling intermediates involved in the induction of IFN- γ . In addition, the same monokines augment IFN- γ gene expression by inducing SET that, in turn, interferes with PP2A catalytic activity, which then reduces dephosphorylation of the proinflammatory signaling intermediates. The resultant direct induction of phosphorylation and reduction of dephosphorylation leads to optimal IFN- γ gene expression after monokine costimulation of NK cells.

As stated, some of these direct and/or indirect PP2A targets are signaling molecules and/or transcription factors that are phosphorylated during monokine activation of NK cells and contribute to the induction of IFN- γ (27–34).

Because induction of PP2A activity by either SET down-regulation or administration of the PP2A activator 1,9-dideoxyforskolin resulted in diminished phosphorylation of ERK, STAT5, STAT4, and RelA (p65), it is plausible that increased PP2A activity limits the induction of IFN- γ gene expression in part by suppressing ERK, STAT5, STAT4, and/or NF- κ B activation. In contrast, a constitutively elevated basal level of SET protein in CD56^{bright} NK cells (and, consequently, lower PP2A activity) is predictably associated with increased amplitude in the phosphorylation/activation of IFN- γ -positive regulators (e.g., ERK1/2) after monokine stimulation of this subset, compared with CD56^{dim} NK cells. Definitive evidence for the *in vivo* relevance of this observation will require additional experimentation.

Although the mechanism whereby SET interferes specifically with PP2A catalytic activity remains unclear, the specificity of its inhibitory activity on PP2A is also supported by its inability to interfere with the function of phosphatases like protein phosphatases 1, 2B, and 2C (20). In addition, the SET protein is also subject to serine phosphorylation, and the significance of this posttranslational modification has not been established (44). SET has been implicated as an inhibitor of granzyme A-dependent mitochondrial damage and apoptosis (45). SET localizes in part to the nucleus, and has been shown to modulate chromatin assembly and gene transcription (44, 46). By using a chemical activator and inhibitors of PP2A activity, as well as by genetically interfering with SET expression, we demonstrate that the effects of SET modulation in NK cells can be reversed through the pharmacologic manipulation of PP2A activity, suggesting that the influence of SET on IFN- γ transcription is largely PP2A dependent. However, we cannot exclude that, in NK cells, these SET-mediated effects are, in part, independent of suppression of PP2A.

The current study implicates the SET–PP2A interplay in the regulation of IFN- γ production, but this is likely only the first example of the role of this signal transduction pathway in NK cell biology. Future studies will explore the preliminary observations mentioned here in regard to the role of SET–PP2A in other NK functions, including cytotoxicity, proliferation, and survival. Furthermore, the regulation of SET–PP2A during NK cell development will need to be investigated. The potential reciprocal interactions between SET–PP2A and other signal transduction pathways (AKT, ERK, JAK/STAT, and NF- κ B) should be addressed at a mechanistic level. For example, we recently reported that PP2A activation leads to the SHP-1-dependent inactivation and proteasome degradation of leukemogenic oncogenes (21). As SET is also overexpressed in various types of cancers (44, 47, 48) and induced by cytokines in normal lymphoid (unpublished data) and myeloid progenitors (21), it is possible that the monokine-induced SET overexpression in NK cells might also have a role in the regulation of the NK cell tumor

surveillance activity. Finally, the genetic manipulation of SET and PP2A within the mouse NK cell lineage, as well as other lymphocyte lineages, will permit investigation of the specific roles of the SET–PP2A interplay in the physiology and pathobiology of the immune system.

MATERIALS AND METHODS

Cells lines and NK cell preparations. The human IL-2–dependent NK cell line NK-92 (a gift from H. Klingemann, Rush Cancer Center, Chicago, IL) was maintained in culture in RPMI 1640 medium (Invitrogen), supplemented with 20% heat-inactivated FBS (Invitrogen), 2 mM L-glutamine, and 150 IU/ml rhIL-2 (Hoffman-LaRoche, Inc.). The amphotropic-packaging cell line Phoenix (a gift from G.P. Nolan, Stanford University, Stanford, CA) was maintained in culture in DME (Invitrogen)/10% FBS medium and grown for 16–18 h to 80% confluence before transfection by calcium phosphate-DNA precipitation (Profection System; Promega). Human NK cells were isolated from peripheral blood leukopacks of healthy individuals (American Red Cross, Columbus, OH) by incubation for 30 min with RosetteSep NK cell antibody cocktail (StemCell Technologies, Inc.), followed by Ficoll-Hypaque density gradient centrifugation and overnight plastic adherence to remove the monocyte population. The fresh NK cell preparations were >85% CD56⁺, as determined by direct immunofluorescence using an anti-CD56 PE-conjugated monoclonal antibody (Immunotech). Alternatively, homogeneous NK cell preparations were obtained from 10-d cocultures of PBL from healthy individuals admixed with irradiated (30 Gy) RPMI 8866 cells (49). NK cell preparations containing >98% CD56⁺ NK cells were obtained by positive selection using CD56 MicroBeads and MACS Separation Columns (Miltenyi Biotech). CD56^{bright} and CD56^{dim} NK cell subsets were purified from fresh blood based on CD56 cell-surface density by FACS upon staining with the anti-CD56-PE antibody (17). All work with human materials was approved by the Institutional Review Board of The Ohio State University Comprehensive Cancer Center.

Retroviral infection of the NK-92 cell line and primary human NK cells. The generation of pSUPER.retro-shSET retroviral vector was previously described (21), as was the GFP-expressing Epstein–Barr virus/retroviral PINCO hybrid vector for human NK cell infection (17, 50). In brief, the shRNA SET constructs were obtained by subcloning the double-stranded 64-mer oligonucleotide containing the SET target sequence (5'-TGAAATAGACAGACTTAAT-3') into the pSUPER.retro.neo+GFP vector (pSUPER; OligoEngine, Inc.). To generate PINCO-SET, the human SET cDNA was obtained from MigRI-FLAG SET (21) by PCR, using a 5' primer containing the BamHI restriction site, the ATG codon, and the nucleotides encoding for the FLAG epitope, and a downstream primer that includes the last nucleotides of SET cDNA linked to an EcoRI restriction site. After BamHI–EcoRI digestion, the FLAG SET product was cloned directionally in the BamHI–EcoRI-digested PINCO (17, 50). For infection, the NK-92 cell line and primary NK cells were first incubated for 2–3 d in the presence of 900 IU/ml IL-2, and then previously published standards (51) were followed. In brief, infectious supernatant from pSUPER, pSUPER.retro-shSET, PINCO, and PINCO-SET retrovirally transfected Phoenix cells were collected after 48 h and used for three cycles of infections. Upon infection, NK-92 cells were sorted (FACSVantage; BD Biosciences) for GFP expression, and primary NK cells were sorted for GFP and CD56 expression upon staining with an anti-CD56 allophycocyanin (APC)-conjugated monoclonal antibody (Immunotech). GFP⁺CD56⁺ primary NK cells were used for experimentation immediately after sorting. Expression of SET mRNA and protein was confirmed in NK-92 cells by real-time RT-PCR and Western blot.

Mice. C57BL/6J mice, which were obtained from The Jackson Laboratory, were 6–8-wk-old female littermates. All animal work was approved by The Ohio State University Animal Care and Use Committee, and mice were treated in accordance with the institutional guidelines for animal care.

Cell culture conditions. Before monokine stimulation, NK-92 cells were cultured in IL-2–free medium containing 10% FBS for 36 h. Cells were next incubated in medium plus 10% FBS at 37°C (10%/ml) for the indicated times, with the addition of various monokines, such as 10 ng/ml IL-12 (Genetics Institute, Inc.), 100 ng/ml IL-18 (R&D Systems), and 100 ng/ml IL-15 (Amgen). Where noted, the PP2A activator 1,9-dideoxy-forskolin or forskolin (Sigma-Aldrich) was added at 40 μ M for 6 or 24 h at 37°C before monokine stimulation. The PP2A inhibitors (25, 37) okadaic acid (20 nM; Calbiochem) and CTS (2 μ g/ml; Peptides International) or vehicle controls (DMSO or water) were added 1 h before stimulation and were present throughout monokine stimulation. The neutralizing anti-human IFN- γ mAb (R&D Systems) was used at 5 μ g/ml.

Western blot analysis. Cells were harvested, washed once with ice-cold PBS, and lysed (10⁸ cells/ml RIPA buffer: 0.15 M NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris, pH 8.0, supplemented with protease and phosphatase inhibitors, 1 mM PMSF, 1 mM Na₃VO₄, 50 mM NaF, 10 mM β -glycerol-phosphate, 1 mM EDTA, and a protease inhibitor cocktail tablet from Roche) as previously described (51). Alternatively, cells were directly lysed in Laemmli buffer (2 \times 10⁵ cells/20 μ l). Western blotting was performed according to previously published protocols (52), and antibody-reactive proteins were detected with horseradish peroxidase-labeled sheep anti-rabbit, -mouse, and/or -goat Ig sera and enhanced chemiluminescence (ECL; GE Healthcare). Proteins were analyzed in 4–15% SDS-PAGE (Bio-Rad Laboratories) using reducing conditions. The mAbs and polyclonal antibodies used were as follows: rabbit polyclonal anti-SET (I2PP2A; Globozymes); goat polyclonal anti-SET (I2PP2A), -pp32 (I1PP2A), and -actin (Santa Cruz Biotechnology); monoclonal anti-PP2Ac, clone 1D6 (Millipore); monoclonal anti-FLAG, clone M2 (Sigma-Aldrich); polyclonal rabbit sera anti-phospho-STAT5^{Tyr694}, -phospho-ERK^{Thr202/Tyr204}, and -phospho-NF-KBp65^{Ser536} (Cell Signaling Technology); polyclonal rabbit sera anti-phospho-STAT4^{Tyr693} (Zymed Laboratories); and monoclonal anti-GRB2 Antibody (Transduction Laboratories).

PP2A phosphatase assay. PP2A assay was performed using the malachite green-based PP2A immunoprecipitation phosphatase assay kit (Millipore), following the manufacturer's protocol. In brief, cells were washed with 0.15 M NaCl and lysate with 1% NP-40, 10 mM Hepes, pH 7.5, 0.15 M NaCl, and 10% glycerol, with protease and without phosphatase inhibitor. 50–100 μ g of proteins were diluted in 50 mM Tris-HCl, pH 7.0, 100 μ M CaCl₂, and PP2A immunoprecipitations were performed using 4 μ g of anti-PP2A antibody and 30 μ l of protein A-agarose beads for sample. Immunoprecipitations were washed and used in the phosphatase reaction following the manufacturer's protocol. The amount of immunoprecipitated PP2A was analyzed by Western blots as an internal control using anti-PP2Ac mAb.

IFN- γ ELISA assays. Quantification of human IFN- γ was performed as previously described (9) using commercially available mAb pairs (Endogen, Inc.). Cell-free supernatants were collected after 18 h of incubation at 37°C. The detection limit was 10–30 pg/ml. Results are shown as the mean of triplicate wells \pm the SEM.

Detection of IFN- γ production by intracellular flow cytometry in monokine-treated mice. C57BL/6J mice were injected i.p. with 200 μ g/mouse of 4% DMSO/PBS or 1,9-dideoxy-forskolin in 4% DMSO/PBS daily for 3 d (21). 1 μ g IL-12 and -18 monokines were injected i.p. 5 h after the last injection of PBS or 1,9-dideoxy-forskolin. Spleens were harvested from C57BL/6J mice 18 h after IL-12 and -18 injection. Splenocytes were either processed immediately for mRNA to quantify IFN- γ by real-time RT-PCR or cultured ex vivo for 4 h with Brefeldin A, harvested, stained with anti-NK1.1 APC and anti-CD3 FITC mAbs (BD Pharmingen), fixed, and permeabilized using Cytotfix/Cytoperm reagent (BD Biosciences). Cells were then stained with an anti-mouse IFN- γ PE mAb or isotype control-PE mAb (BD Pharmingen). Cells were assessed on a FACSCalibur cytometer (BD Biosciences), and analyses were performed using the CellQuest (BD Biosciences) software program.

Real-time RT-PCR. Total mRNA was extracted using RNeasy Mini kits (Qiagen) and cDNA was generated according to the manufacturer's recommendations (Invitrogen). Real-time RT-PCR reactions for IFN- γ transcripts were performed as a multiplex reaction with a primer/probe set specific for the human IFN- γ mRNA, as previously described (17). cDNA from PHA-activated human lymphocytes served as positive controls for cytokine transcripts, and water (no template) was used as a negative control. The human SET primers and probe used are as follows: forward primer 5'-AAAAGGAGCT-CAGAAAAGAAGACAGCA-3', reverse primer 5'-CATCAATGTGTTCAA-TCGCT-3', and probe 5'-CCGACGAGACCTCAGAAAAGAAGACACA-3'. cDNA samples were used as a template in a PCR-amplification reaction containing a set of primers and a FAM-labeled probe for the SET transcripts (1), or a set of primers and a VIC-labeled probe for 18S (2). Reactions were performed using an ABI prism 7700 sequence detector (Taqman; Applied Biosystems), and data were analyzed with the Sequence Detector version 1.6 software to establish the PCR cycle at which the fluorescence exceeded a set threshold, C_T , for each sample. Data were analyzed according to the comparative C_T method, as previously described (3), using the internal control 18S transcript levels to normalize differences in sample loading and preparation. Results represent the n -fold difference of transcript levels in a particular sample compared with calibrator cDNA (cDNA samples of unstimulated PINCO-infected, pSUPER, retro-infected, or NK-92 cells). Results are expressed as the mean \pm the SEM of triplicate reaction wells.

Cytotoxicity assays. Before the assay, NK-92 effectors cells were cultured in IL-2-free medium containing 10% FBS for 36 h. K562 were used as target in a 3-h ^{51}Cr release assay (31). A constant number of target cells (10^4 cells/well) and serial dilution of effectors cells were used in triplicates. Spontaneous release was always $<10\%$.

Statistics. For each statistical comparison, a one-sample t test was performed. Each data point for the experiment under consideration was generated from a process that involved a comparison to a control condition. That is, each control group value is normalized to 1, and the experimental treatment resulted in a data point that was either $<$ or >1 . If necessary for the assumption of normality, the data were natural log transformed. A one-sample t test was performed with the α set to 0.05.

Online supplemental material. Fig. S1 shows that single-monokine (IL-12, -15, or -18) stimulation of primary NK cells induces SET expression. Fig. S2 shows that, in primary NK cells, the IL-12/-18-induced up-regulation of SET is not altered by neutralizing IFN- γ . Fig. S3 shows that downmodulation of SET diminishes NK cell cytotoxicity, whereas its overexpression promotes NK cell cytotoxicity, and that pharmacological activation of PP2A inhibits NK cell cytotoxicity in SET-overexpressing cells. The online version of this article is available at <http://www.jem.org/cgi/content/full/jem.20070419/DC1>.

We thank Ms. Jing Wen for technical assistance.

This work was supported in part by National Cancer Institute grants CA16058 (M.A. Caligiuri), CA95426 (M.A. Caligiuri), CA68458 (M.A. Caligiuri), and CA095512 (D. Perrotti). P. Neviani is a Fellow of the American Italian Cancer Foundation. D. Perrotti is a Scholar of the Leukemia and Lymphoma Society of America.

The authors have no conflicting financial interest.

Submitted: 28 February 2007

Accepted: 29 August 2007

REFERENCES

- Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187–376.
- Farag, S.S., T.A. Fehniger, L. Ruggeri, A. Velardi, and M.A. Caligiuri. 2002. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood.* 100:1935–1947.
- Fehniger, T.A., M.H. Shah, M.J. Turner, J.B. VanDeusen, S.P. Whitman, M.A. Cooper, K. Suzuki, M. Wechsler, F. Goodsaid, and M.A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162:4511–4520.
- Raulet, D.H. 2004. Interplay of natural killer cells and their receptors with the adaptive immune response. *Nat. Immunol.* 5:996–1002.
- Zitvogel, L., M. Terme, C. Borg, and G. Trinchieri. 2006. Dendritic cell-NK cell cross-talk: regulation and physiopathology. *Curr. Top. Microbiol. Immunol.* 298:157–174.
- Martin-Fontecha, A., L.L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN- γ for T(H)1 priming. *Nat. Immunol.* 5:1260–1265.
- Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri. 2001. The biology of human natural killer-cell subsets. *Trends Immunol.* 22:633–640.
- Fehniger, T.A., M.A. Cooper, G.J. Nuovo, M. Cella, F. Facchetti, M. Colonna, and M.A. Caligiuri. 2003. CD56bright natural killer cells are present in human lymph nodes and are activated by T cell-derived IL-2: a potential new link between adaptive and innate immunity. *Blood.* 101:3052–3057.
- Cooper, M.A., T.A. Fehniger, S.C. Turner, K.S. Chen, B.A. Ghaeheri, T. Ghayur, W.E. Carson, and M.A. Caligiuri. 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood.* 97:3146–3151.
- Lanier, L.L., A.M. Le, C.I. Civin, M.R. Loken, and J.H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* 136:4480–4486.
- Lieberman, L.A., and C.A. Hunter. 2002. Regulatory pathways involved in the infection-induced production of IFN- γ by NK cells. *Microbes Infect.* 4:1531–1538.
- Kaplan, D.H., V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, and R.D. Schreiber. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc. Natl. Acad. Sci. USA.* 95:7556–7561.
- Shankaran, V., H. Ikeda, A.T. Bruce, J.M. White, P.E. Swanson, L.J. Old, and R.D. Schreiber. 2001. IFN γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature.* 410:1107–1111.
- Shtrichman, R., and C.E. Samuel. 2001. The role of gamma interferon in antimicrobial immunity. *Curr. Opin. Microbiol.* 4:251–259.
- Schroder, K., P.J. Hertzog, T. Ravasi, and D.A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* 75:163–189.
- Lieberman, L.A., and C.A. Hunter. 2002. The role of cytokines and their signaling pathways in the regulation of immunity to *Toxoplasma gondii*. *Int. Rev. Immunol.* 21:373–403.
- Trotta, R., R. Parihar, J. Yu, B. Becknell, J. Allard II, J. Wen, W. Ding, H. Mao, S. Tridandapani, W.E. Carson, and M.A. Caligiuri. 2005. Differential expression of SHIP1 in CD56bright and CD56dim NK cells provides a molecular basis for distinct functional responses to monokine costimulation. *Blood.* 105:3011–3018.
- Yu, J., M. Wei, B. Becknell, R. Trotta, S. Liu, Z. Boyd, M.S. Jaung, B.W. Blaser, J. Sun, D.M. Benson Jr., et al. 2006. Pro- and antiinflammatory cytokine signaling: reciprocal antagonism regulates interferon-gamma production by human natural killer cells. *Immunity.* 24:575–590.
- Janssens, V., and J. Goris. 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* 353:417–439.
- Li, M., A. Makkinje, and Z. Damuni. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J. Biol. Chem.* 271:11059–11062.
- Neviani, P., R. Santhanam, R. Trotta, M. Notari, B.W. Blaser, S. Liu, H. Mao, J.S. Chang, A. Galiotta, A. Uttam, et al. 2005. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell.* 8:355–368.
- Fan, Z., P.J. Beresford, D.Y. Oh, D. Zhang, and J. Lieberman. 2003. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell.* 112:659–672.

23. Seo, S.B., P. McNamara, S. Heo, A. Turner, W.S. Lane, and D. Chakravarti. 2001. Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell*. 104:119–130.
24. Millward, T.A., S. Zolnierowicz, and B.A. Hemmings. 1999. Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem. Sci.* 24:186–191.
25. Sontag, E. 2001. Protein phosphatase 2A: the Trojan Horse of cellular signaling. *Cell. Signal*. 13:7–16.
26. Kawada, M., M. Kawatsu, T. Masuda, S. Ohba, M. Amemiya, T. Kohama, M. Ishizuka, and T. Takeuchi. 2003. Specific inhibitors of protein phosphatase 2A inhibit tumor metastasis through augmentation of natural killer cells. *Int. Immunopharmacol.* 3:179–188.
27. Bream, J.H., D.L. Hodge, R. Gonsky, R. Spolski, W.J. Leonard, S. Krebs, S. Targan, A. Morinobu, J.J. O'Shea, and H.A. Young. 2004. A distal region in the interferon- γ gene is a site of epigenetic remodeling and transcriptional regulation by interleukin-2. *J. Biol. Chem.* 279:41249–41257.
28. Kalina, U., D. Kauschat, N. Koyama, H. Nuernberger, K. Ballas, S. Koschmieder, G. Bug, W.K. Hofmann, D. Hoelzer, and O.G. Ottmann. 2000. IL-18 activates STAT3 in the natural killer cell line 92, augments cytotoxic activity, and mediates IFN- γ production by the stress kinase p38 and by the extracellular regulated kinases p44erk-1 and p42erk-2. *J. Immunol.* 165:1307–1313.
29. Sica, A., L. Dorman, V. Viggiano, M. Cippitelli, P. Ghosh, N. Rice, and H.A. Young. 1997. Interaction of NF- κ B and NFAT with the interferon- γ promoter. *J. Biol. Chem.* 272:30412–30420.
30. Strengell, M., S. Matikainen, J. Siren, A. Lehtonen, D. Foster, I. Julkunen, and T. Sareneva. 2003. IL-21 in synergy with IL-15 or IL-18 enhances IFN- γ production in human NK and T cells. *J. Immunol.* 170:5464–5469.
31. Trotta, R., K.A. Puorro, M. Paroli, L. Azzoni, B. Abebe, L.C. Eisenlohr, and B. Perussia. 1998. Dependence of both spontaneous and antibody-dependent, granule exocytosis-mediated NK cell cytotoxicity on extracellular signal-regulated kinases. *J. Immunol.* 161:6648–6656.
32. Akira, S. 2000. The role of IL-18 in innate immunity. *Curr. Opin. Immunol.* 12:59–63.
33. Barbulescu, K., C. Becker, J.F. Schlaak, E. Schmitt, K.H. Meyer zum Buschenfelde, and M.F. Neurath. 1998. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN- γ promoter in primary CD4+ T lymphocytes. *J. Immunol.* 160:3642–3647.
34. Thierfelder, W.E., J.M. van Deursen, K. Yamamoto, R.A. Tripp, S.R. Sarawar, R.T. Carson, M.Y. Sangster, D.A. Vignali, P.C. Doherty, G.C. Grosveld, and J.N. Ihle. 1996. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature*. 382:171–174.
35. Gong, J.H., G. Maki, and H.G. Klingemann. 1994. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia*. 8:652–658.
36. Feschenko, M.S., E. Stevenson, A.C. Nairn, and K.J. Swadner. 2002. A novel cAMP-stimulated pathway in protein phosphatase 2A activation. *J. Pharmacol. Exp. Ther.* 302:111–118.
37. Kawada, M., M. Amemiya, M. Ishizuka, and T. Takeuchi. 1999. Cytostatin, an inhibitor of cell adhesion to extracellular matrix, selectively inhibits protein phosphatase 2A. *Biochim. Biophys. Acta*. 1452:209–217.
38. Dunn, G.P., L.J. Old, and R.D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity*. 21:137–148.
39. Bouma, G., and W. Strober. 2003. The immunological and genetic basis of inflammatory bowel disease. *Nat. Rev. Immunol.* 3:521–533.
40. Becknell, B., T.L. Hughes, A.G. Freud, B.W. Blaser, J. Yu, R. Trotta, H.C. Mao, M.L. Caligiuri de Jesus, M. Alghothani, D.M. Benson Jr., et al. 2006. The Hlx homeobox transcription factor negatively regulates interferon- γ production in monokine-activated natural killer cells. *Blood*. 109:2481–2487.
41. Yang, J., G.H. Fan, B.E. Wadzinski, H. Sakurai, and A. Richmond. 2001. Protein phosphatase 2A interacts with and directly dephosphorylates RelA. *J. Biol. Chem.* 276:47828–47833.
42. Yokoyama, N., N.C. Reich, and W.T. Miller. 2001. Involvement of protein phosphatase 2A in the interleukin-3-stimulated Jak2-Stat5 signaling pathway. *J. Interferon Cytokine Res.* 21:369–378.
43. Letourneux, C., G. Rocher, and F. Porteu. 2006. B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. *EMBO J.* 25:727–738.
44. Adachi, Y., G.N. Pavlakis, and T.D. Copeland. 1994. Identification and characterization of SET, a nuclear phosphoprotein encoded by the translocation break point in acute undifferentiated leukemia. *J. Biol. Chem.* 269:2258–2262.
45. Martinvalet, D., P. Zhu, and J. Lieberman. 2005. Granzyme A induces caspase-independent mitochondrial damage, a required first step for apoptosis. *Immunity*. 22:355–370.
46. Gamble, M.J., H. Erdjument-Bromage, P. Tempst, L.P. Freedman, and R.P. Fisher. 2005. The histone chaperone TAF-I/SET/INHAT is required for transcription in vitro of chromatin templates. *Mol. Cell. Biol.* 25:797–807.
47. Carlson, S.G., E. Eng, E.G. Kim, E.J. Perlman, T.D. Copeland, and B.J. Ballermann. 1998. Expression of SET, an inhibitor of protein phosphatase 2A, in renal development and Wilms' tumor. *J. Am. Soc. Nephrol.* 9:1873–1880.
48. Fornerod, M., J. Boer, S. van Baal, M. Jaegle, M. von Lindern, K.G. Murti, D. Davis, J. Bonten, A. Buijs, and G. Grosveld. 1995. Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene*. 10:1739–1748.
49. Perussia, B., and M.J. Loza. 2005. Purification of peripheral blood natural killer cells. *Methods Mol. Med.* 107:147–161.
50. Becknell, B., R. Trotta, J. Yu, W. Ding, H.C. Mao, T. Hughes, T. Marburger, and M.A. Caligiuri. 2005. Efficient infection of human natural killer cells with an EBV/retroviral hybrid vector. *J. Immunol. Methods*. 296:115–123.
51. Trotta, R., T. Vignudelli, O. Candini, R.V. Intine, L. Pecorari, C. Guerzoni, G. Santilli, M.W. Byrom, S. Goldoni, L.P. Ford, et al. 2003. BCR/ABL activates mdm2 mRNA translation via the La antigen. *Cancer Cell*. 3:145–160.
52. Trotta, R., P. Kanakaraj, and B. Perussia. 1996. Fc γ R-dependent mitogen-activated protein kinase activation in leukocytes: a common signal transduction event necessary for expression of TNF- α and early activation genes. *J. Exp. Med.* 184:1027–1035.