JCI The Journal of Clinical Investigation

Tyrosine kinase pathways modulate tumor susceptibility to natural killer cells

Roberto Bellucci, Hong-Nam Nguyen, Allison Martin, Stefan Heinrichs, Anna C. Schinzel, William C. Hahn, Jerome Ritz

J Clin Invest. 2012;122(7):2369-2383. https://doi.org/10.1172/JCI58457.

Research Article **Oncology**

Natural killer (NK) cells are primary effectors of innate immunity directed against transformed tumor cells. In response, tumor cells have developed mechanisms to evade NK cell–mediated lysis through molecular mechanisms that are not well understood. In the present study, we used a lentiviral shRNA library targeting more than 1,000 human genes to identify 83 genes that promote target cell resistance to human NK cell–mediated killing. Many of the genes identified in this genetic screen belong to common signaling pathways; however, none of them have previously been known to modulate susceptibility of human tumor cells to immunologic destruction. Gene silencing of two members of the JAK family (JAK1 and JAK2) increased the susceptibility of a variety of tumor cell types to NK-mediated lysis and induced increased secretion of IFN-γ by NK cells. Treatment of tumor cells with JAK inhibitors also increased susceptibility to NK cell activity. These findings may have important clinical implications and suggest that small molecule inhibitors of tyrosine kinases being developed as therapeutic antitumor agents may also have significant immunologic effects in vivo.

Find the latest version:

https://jci.me/58457/pdf

Tyrosine kinase pathways modulate tumor susceptibility to natural killer cells

Roberto Bellucci,[1,2,3] Hong-Nam Nguyen,[1] Allison Martin,[1] Stefan Heinrichs,[3,4] Anna C. Schinzel,[1,5] William C. Hahn,[1,2,3,5] and Jerome Ritz[1,2,3,6]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute (DFCI), Boston, Massachusetts, USA. [2]Department of Medicine, Brigham and Woman's Hospital, Boston, Massachusetts, USA. [3]Harvard Medical School, Boston, Massachusetts, USA. [4]Department of Pediatric Oncology, [5]Center for Cancer Genome Discovery, and [6]Cancer Vaccine Center, DFCI, Boston, Massachusetts, USA.

Natural killer (NK) cells are primary effectors of innate immunity directed against transformed tumor cells. In response, tumor cells have developed mechanisms to evade NK cell–mediated lysis through molecular mechanisms that are not well understood. In the present study, we used a lentiviral shRNA library targeting more than 1,000 human genes to identify 83 genes that promote target cell resistance to human NK cell–mediated killing. Many of the genes identified in this genetic screen belong to common signaling pathways; however, none of them have previously been known to modulate susceptibility of human tumor cells to immunologic destruction. Gene silencing of two members of the JAK family (JAK1 and JAK2) increased the susceptibility of a variety of tumor cell types to NK-mediated lysis and induced increased secretion of IFN-γ by NK cells. Treatment of tumor cells with JAK inhibitors also increased susceptibility to NK cell activity. These findings may have important clinical implications and suggest that small molecule inhibitors of tyrosine kinases being developed as therapeutic antitumor agents may also have significant immunologic effects in vivo.

Introduction

Natural killer (NK) cells are a critical component of the innate immune response against infectious pathogens and malignant transformation (1, 2). NK cells mediate this activity through the elaboration of various cytokines as well as through direct cytolytic activity. However, unlike adaptive immune cells, which utilize specific clonal recognition receptors, NK cell activation depends on a complex balance between activating and inhibitory signals (3, 4). In patients with cancer, it is presumed that tumor cells have developed mechanisms to suppress NK cell activation and resist lysis by endogenous NK cells, but the molecular basis for target resistance is not well understood.

RNAi has made it possible to perform loss-of-function genetic analysis in mammalian cells, and the development of genome-wide shRNA libraries has facilitated large-scale unbiased screens. These libraries have been successfully used to identify novel mechanisms of cell transformation (5–7), as well as to identify genes that play important roles in cancer progression in different tumors (8, 9). Many of these basic discoveries will have clinical significance, facilitating the discovery of genes and pathways that can be effectively targeted by new specific inhibitory drugs.

We hypothesized that this approach could also be used to identify molecular pathways that modulate tumor cell susceptibility to the innate immune system. To test this hypothesis, we designed an shRNA screen to monitor interactions between IM-9, a multiple myeloma (MM) tumor cell target, and NKL, a functional human NK cell line (10). IM-9 myeloma target cells were transduced with the TRC1 kinase/phosphatase subset of the TRC1 shRNA lentivirus library developed at The RNAi Consortium (TRC) (6, 11). shRNA-expressing IM-9 cells were subsequently incubated with NKL effector cells, and the strength of this interaction was assessed by measuring IFN-γ release from NKL cells. Using this approach, we identified a set of 83 genes that when silenced increased the susceptibility of IM-9 tumor cells to NK cell activity. Remarkably, many of the genes identified in this screen belong to common intracellular signaling pathways such as MAPK, PIK3, IGF1R, JAK1, and JAK2. These pathways are known to be involved in a variety of cellular functions and often integrate signals resulting from membrane receptor-ligand interactions (12, 13).

To validate the results of the shRNA screen, we established a panel of independent target cell lines expressing individual shRNAs. In almost all instances, effective reduction of specific protein expression resulted in enhanced sensitivity of the tumor cell target to NK activity. Moreover, specific kinase inhibition with small molecules had similar effects on susceptibility to human NK cells in vitro. This study provides evidence that common signaling pathways can regulate susceptibility of human tumor cells to killing by immunologic effector cells. These finding may also have important clinical implications and suggest that small molecule inhibitors of these kinases that are being developed as direct therapeutic antitumor agents may also have important immunologic effects in vivo.

Results

High-throughput shRNA screen. To identify pathways that modulate the susceptibility of human tumor cells to NK cells, we designed a high-throughput genetic screen to measure this specific cell-cell interaction. In this assay, IM-9 myeloma cells were transduced in 5 replicate sets with individual lentiviruses expressing shRNAs from the phosphatase/kinase subset of the TRC library (Figure 1A). Viability was assessed in 2 replicates; one was treated with puromycin for selection and one left without puromycin. After B-score normalization (14), all shRNA/wells with less than 70% viability in either set were excluded from subsequent analysis. This excluded individual vectors with low transduction efficiency and vectors that induced toxicity in the absence of puromycin. NKL effector

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J Clin Invest*. 2012;122(7):2369–2383. doi:10.1172/JCI58457.

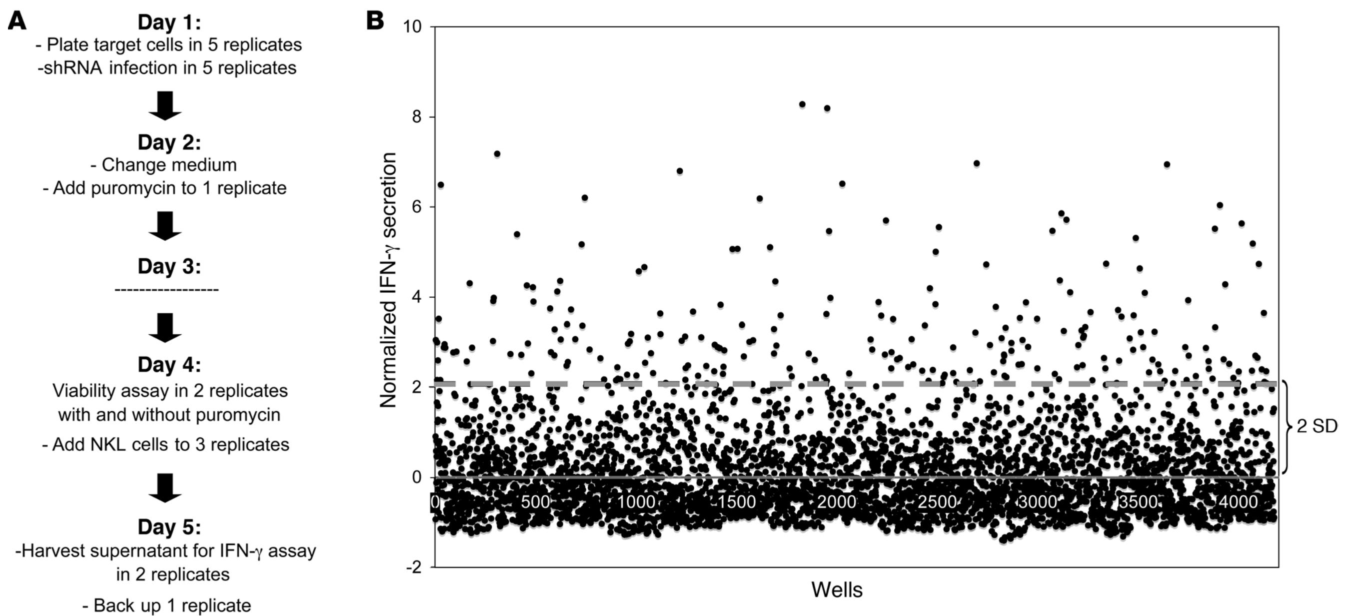


Figure 1
Genetic screen to identify modulators of susceptibility to human NK cells. (**A**) Schematic representation of the 5-day workflow. (**B**) Normalized distribution of the levels of IFN-γ secretion by NKL cells for 4,177 selected shRNAs.

cells were added to the 3 remaining replicate sets, and supernatants were subsequently harvested for measurement of IFN-γ secretion. Analysis of 2 replicates with NKL cells (12,288 wells) showed significant reproducibility (r = 0.858) of IFN-γ measurements (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58457DS1). Mean levels of IFN-γ in 4,177 evaluable wells were normalized using z-score analysis and plotted as shown in Figure 1B. Only wells with IFN-γ values in the top fifth percentile, which corresponded to 2 standard deviations above the mean z-score ($z = 0.108 \pm 1.09$), were considered to demonstrate significantly increased levels of IFN-γ secretion. These 209 hits corresponded to 192 individual genes, since some had more than 1 shRNA scoring in the top 5%. To reduce the likelihood of identifying off-target shRNA effects (15, 16), only genes that were identified by 2 or more independent shRNAs, with the second shRNA at least in the top 20%, were selected for further analysis. With this ranking, a total of 83 genes (Table 1) were considered positive. Supernatants from the third replicate were kept as a backup.

To validate the reproducibility of our screening strategy, we randomly selected 1 of the 16 TRC1 library plates (K40) and independently repeated the entire screen using different batches of IM-9 and NKL cells. IFN-γ measurements were compared with the values obtained in the original screen, and the correlation coefficient between the 2 screens was 0.766, indicating highly significant reproducibility of the experimental setting (Supplemental Figure 2).

Signaling pathways involved in tumor susceptibility to NKL effector cells. As shown in Table 1, we identified 83 genes that, when silenced in tumor cell targets, resulted in increased IFN-γ secretion from NKL effector cells. The TRC library subset used in this study consisted of 1,028 genes, including 476 protein kinases (46% of the 1,028), 180 phosphatases (17%), and 372 genes with different functions (36%). Interestingly, of the 83 genes selected, 66 (79%) were kinases, 12 (14.4%) were proteins with non-kinase functions, and only 4 (4.8%) were phosphatases. Many of these protein kinases were associated with common signaling pathways, suggesting that activation of these pathways at different levels can mediate susceptibility of tumor cells to human NK cells. The MAPK pathway was the most highly represented, with 15 genes (18% of the 83 genes and 22% of the 66 kinases identified), while the AKT/PIK3 and the CDK pathways were represented by 3 and 6 genes, respectively. The MAPK and PIK3 pathways regulate a variety of cellular functions including cell cycle progression, cell survival, angiogenesis, and cell migration (17). Activation of these intracellular pathways is linked to surface membrane receptors, and 14 cell surface receptors or membrane-associated genes (16.8% of total) were also identified. This group included 3 members of the TGF-β family (*ACVRL1*, *ACVR1*, *ACVR1B*), 1 member of the ephrin receptor family (*EPHA6*), 3 receptor tyrosine kinases (*IGF1R*, *INSR*, and *LTK*), and 2 members of the JAK family kinases (*JAK1* and *JAK2*) that are associated with several membrane cytokine receptors (18).

Validation of selected genes representing different signaling pathways. To validate our experimental approach, we selected 5 genes listed in Table 1 for further detailed characterization. These included *MAPK1*, 2 membrane receptors (*IGF1R* and *INSR*), and 2 members of the JAK family (*JAK1* and *JAK2*). For each of these genes, we established a series of puromycin-resistant independent IM-9 cell lines with stable expression of a specific shRNAs (at least 3 different sequences per target gene) or irrelevant control shRNAs. The target sequences of the specific shRNAs and irrelevant control shRNAs used to knock down gene expression in tumor cell lines are summarized in Supplemental Tables 1 and 2. Each genetically modified cell line was tested for downregulation of the target protein by Western blotting or flow cytometry, and the level of protein expression was correlated with susceptibility to NK-92 cells, an additional NK effector cell line (19), as well as to NKL cells.

Table 1

Eighty-three selected genes that modulate tumor cell susceptibility to NKL cells

	Membrane-associated proteins							Intracellular proteins				
TGF-β family	**Ephrin**	**IGF1R/INSR**	**Membrane-associated**	**Others**	**PI3K/Akt**	**MAPK**	**CDK**	**Cytoskeletal function**	**DNA/RNA binding**	**Tumor suppressor oncogenes**	**Kinases with other function**	**Kinases with unknown function**
ACVRL1	*EPHA6*	*IGF1R*	*JAK1*	*CDH1*	*PIK3CA*	*MAPK1*	*CDKN2D*	*CDC42BPG*	*MSH2*	*SKI*	*PRKAA2*	*LOC440354*
ACVR1		*INSR*	*JAK2*		*PIK3CB*	*MAP3K10*	*CDKN2A*	*ROCK1*	*POT1*	*PRKCA*	*CSK*	*LOC442370*
ACVR1C		*LTK*	*DOCK4*		*CSNK2A1*	*MAP4K2*	*CDK5R2*	*PARG1*	*CIB3*		*PRKCABP*	*LOC401313*
			TRAD			*MAPK12*	*SKP2*	*OXSR1*	*TLK2*		*TP53RK*	*LOC389873*
			SHH			*MAPK10*	*CDK15*	*CASK*	*PRKRA*		*ZC3HC1*	*LOC343474*
			HSPA5			*RPS6KA5*	*CDK18*	*APEG1*	*DTYMK*		*GALK1*	*FLJ25006*
						TAOK1			*NEK11*		*CKMT2*	*TEX14*
						TAOK3			*TRIM27*		*STK25*	*PKN2*
						MKNK2			*ZCWCC3*		*STK11IP*	*PSKH2*
						TOPK			*SYT5*		*MELK*	
						RPH3A			*SYT14L*		*PKLR*	
						ICK			*SYT17*		*CLK3*	
						TRIB1					*TK2*	
						CAMK1					*LRRK1*	
						MAP3K5					*LRRK2*	
											PFKP	

Three independent shRNAs targeting MAPK1/ERK2 (MAPK1-1, MAPK1-2, MAPK1-5) induced increased IFN-γ secretion by NKL cells in our initial screen. IM-9 cell lines expressing each of these shRNAs were compared with parental unmodified IM-9 and IM-9 cells expressing control shRNAs (shCTRL-1 and shCTRL-2). All cell lines expressing shRNAs maintained excellent viability and proliferative capacity in vitro after puromycin selection. As shown in Figure 2, A and B, the shRNAs that induced the strongest downregulation of MAPK1-p42 protein expression in IM-9 cells as measured by Western blot analysis also induced the greatest increase in IFN-γ secretion by both NKL and NK-92 effector cells. IM-9 cells infected with 2 irrelevant shRNAs had no effect on MAPK1-p42 protein expression or IFN-γ secretion by NK effector cells. Similar results were obtained with shRNAs targeting JAK1 and JAK2. Two shRNAs targeting JAK1 (Jak1-1 and Jak1-3) effectively reduced protein levels in IM-9 cells, but one JAK1-targeting shRNA (Jak1-2) had no effect (Figure 2B). Similarly, 2 shRNAs targeting JAK2 (Jak2-3 and Jak2-4) effectively reduced protein levels in IM-9 cells, and one JAK2-targeting shRNA (Jak2-2) had no effect (Figure 2B). As shown in Figure 2A, only those JAK1- and JAK2-specific shRNAs that reduced protein expression in IM-9 cells induced increased IFN-γ secretion when these cells were incubated with either NKL or NK-92 effector cells.

We next examined 2 transmembrane proteins, IGF1R and INSR. IGF1R, a tyrosine kinase receptor, has been identified as a target for cancer therapy (20), and several studies have shown that binding of IGF to IGF1R can induce phosphorylation of RAF1 and PI3K, resulting in downstream activation of MAPK and PI3K/Akt pathways (21). Our screen identified two shRNAs (IGF1R-1 and IGF1R-3) that induced increased IFN-γ secretion from NKL cells and one shRNA (IGF1R-4) that had no effect. Incubation of NKL and NK-92 effector cells with IM-9 cells expressing shRNAs IGF1R-1 and IGF1R-3 induced 48% and 60% more IFN-γ secretion by NKL and 35% and 40% more IFN-γ secretion by NK-92 when compared with a control shRNA. There was no difference in the level of IFN-γ secreted by both NKL and NK-92 cells when IM-9 cells expressing shRNA IGF1R-4 were used (Figure 2A). Analysis of IGF1R protein levels by flow cytometry confirmed the specific downregulation of IGF1R protein by shRNA IGF1R-1 and IGF1R-3, while IGF1R protein levels were not affected by shRNA IGF1R-4 (Figure 2C).

Three different shRNAs for INSR were also tested. IM-9 cells expressing shRNA INSR-3 induced higher levels of IFN-γ secretion by both NKL and NK-92 cells, and this correlated well with reduced levels of INSR as determined by flow cytometry (Figure 2, A and C). INSR-1 shRNA had very little effect on IFN-γ secretion by NKL and NK-92 cells and did not reduce INSR protein levels. However, the third shRNA tested (INSR-4) resulted in a significant increase in IFN-γ secretion by both NKL and NK-92 cells in independent experiments, but this did not correlate with any change in INSR protein levels in IM-9 cells (Figure 2, A and C). Of 15 shRNAs that were tested individually in IM-9 cells, INSR-4 is the only sequence that gave discordant results, and the effect of this shRNA on protein expression could not be correlated with increased secretion of IFN-γ by both NKL and NK-92 effector cells.

Figure 2

Validation of selected genes in IM-9 target cells with stable expression of individual shRNAs. IM-9 cells expressing 3 different shRNAs targeting MAPK1, JAK1, JAK2, IGF1R, and INSR were incubated overnight with NKL and NK-92 effector cells at a 1:1 E/T ratio. (**A**) Increased IFN-γ secretion induced in NKL or NK-92 compared with IM-9 target cells incorporating an irrelevant shRNA control. (**B**) Specific downregulation of MAPK1, JAK1, and JAK2 proteins by individual shRNAs was analyzed by Western blotting. (**C**) Specific downregulation of IGF1R and INSR by individual shRNAs was analyzed by flow cytometry. PE-CTRL, PE-conjugated secondary antibody alone.

Differential role of JAK family genes in tumor cell resistance to NK cells. Two of the genes that had the strongest effect on NK cell activity were members of the JAK family of kinases. This effect was only noted for JAK1 and JAK2, while none of the shRNAs targeting other members of this family (JAK3 and TYK2) induced similar activity. As shown in Figure 3, A and B, IM-9 cell lines, each expressing different shRNAs targeting JAK3 and TYK2, were tested for expression of JAK3 and TYK2 and their ability to activate NKL and NK-92 cells. Three of 4 JAK3 shRNAs (Jak3-1, Jak3-2, and Jak3-3) and 2 of 4 TYK2 shRNAs (TYK2-1 and TYK2-4) successfully reduced expression of the target protein, but none of these shRNAs induced increased secretion of IFN-γ from either NKL or NK-92 effector cells. These results confirmed that specific downregulation of JAK1 and JAK2 but not JAK3 or TYK2 could modulate tumor cell susceptibility to NK cell activity.

To examine the specificity of JAK1 inhibition on susceptibility to NK cell activity, we undertook further characterization of IM-9 target cells expressing each of the 3 JAK1 shRNA vectors. As shown in Figure 4A, JAK1 protein expression was reduced in IM-9 cells expressing Jak1-1 and Jak1-3 shRNAs. These effects were specific for JAK1, and expression of JAK2, JAK3, and TYK2 was not reduced. Similarly, quantitative RT-PCR demonstrated reduced levels of *JAK1* mRNA in these cell lines (Figure 4B). As shown in Figure 4C, reduced expression of JAK1 resulted in significantly higher levels of IFN-γ secretion by NKL and NK-92 effector cells. Intracellular staining confirmed that IFN-γ was derived from NK effector cells (Supplemental Figure 3). In conventional cytotoxicity assays, IM-9 cells with reduced expression of JAK1 were more susceptible to lysis by both NKL and NK-92 effector cells when compared with IM-9 cells infected with a control shRNA (shCTRL-2) (Figure 4D). No difference in cytotoxicity was noted in IM-9 cells expressing shRNA Jak1-2 that had not affected JAK1 protein expression. Increased killing of JAK1-knockout IM-9 cells (IM-9-JAK1-KO cells) by NK cells was also confirmed using an Annexin V assay we developed to quantify the induction of apoptosis in target cells incubated with NK effector cells. In this assay, effector cells were incubated with target cells at a 1:1 effector/target (E/T) ratio for a 12-hour period. As shown in Figure 4E, IM-9 cells lacking expression of JAK1 (Jak1-1 and Jak1-3 shRNAs) underwent significantly more apoptosis than IM-9 cells infected with a control hairpin or with a JAK1 shRNA that does not reduce JAK1 expression (Jak1-2 shRNA). Increased apoptosis

Figure 3

Analysis of IM-9 cells expressing shRNAs targeting TYK2 and JAK3. (**A**) Effects of 4 shRNAs targeting TYK2 on levels of IFN-γ secretion by NKL or NK-92 effector cells incubated with IM-9-TYK2 at a 1:1 E/T ratio. (**B**) Effects of 4 shRNAs targeting JAK3 on levels of IFN-γ secretion by NKL or NK-92 effector cells incubated with IM-9-JAK3 at a 1:1 E/T ratio. Data represent the mean of 4 independent experiments with each target tested in duplicate. (**C**) Protein levels in each target cell line were evaluated by Western blotting using anti-TYK2 and anti-JAK3 specific antibodies.

was observed when IM-9 cells were incubated with either NKL or NK-92 effector cells, but the level of spontaneous apoptosis for IM-9 cells expressing each of the JAK1 shRNAs was always less than 7% if no effector cells were present.

Results of similar experiments carried out with 3 shRNAs specific for JAK2 are summarized in Figure 5. Western blot analysis and quantitative RT-PCR confirmed that IM-9 cells expressing Jak2-3 and Jak2-4 expressed lower levels of JAK2, and expression of these shRNAs did not affect expression of the other members of the JAK family (Figure 5, A and B). In multiple independent experiments, only IM-9 cells that expressed lower levels of JAK2 induced higher levels of IFN-γ secretion (Figure 5C), and this correlated closely with increased lysis (Figure 5D) and induction of apoptosis by both NKL and NK-92 effector cells (Figure 5E).

Increased susceptibility of target cells with reduced expression of JAK1 and JAK2 to primary human NK cells. PBMCs from 4 normal donors were used as a source of primary NK cells and incubated with IM-9 cells expressing various JAK1- and JAK2-targeting shRNAs at different E/T ratios. As shown in Figure 6A, IM-9 cells expressing 2 specific shRNAs (Jak1-1 and Jak1-3) induced increased secretion of IFN-γ by PBMCs when compared with 3 control shRNAs and IM-9 parental cells (P = 0.034 and P = 0.0088, Jak1-1-KO vs. shCTRL-2 and Jak1-3-KO vs. shCTRL-2, respectively, at a 5:1 E/T ratio; and P = 0.012 and P = 0.0057, Jak1-1-KO vs. shCTRL-2 and Jak1-3-KO vs shCTRL-2, respectively, at a 10:1 E/T ratio). Cells expressing Jak1-2 shRNA, which was not effective in silencing JAK1, did not induce increased sensitivity to PBMCs. Primary NK cells were also more effective when tested against IM-9 cells with reduced expression of JAK2. As shown in Figure 6B, IM-9 cells expressing 2 shRNAs (Jak2-3 and Jak2-4) that efficiently silenced JAK2 were more susceptible to lysis when compared with the 3 controls and IM-9 parental cells (P = 0.009 and P = 0.012, Jak2-3-KO vs. shCTRL-2 and Jak2-4 vs. shCTRL-2, respectively, at a 5:1 E/T ratio; and P = 0.034 and P = 0.036, Jak2-3-KO vs. shCTRL-2 and Jak2-4-KO vs. shCTRL-2, respectively, at a 10:1, E/T ratio). To confirm that the increased susceptibility of IM-9-JAK1-KO and IM-9-JAK2-KO cells was NK specific, we repeated these experiments using purified NK cells. Purified NK cells (>90% $CD56^+CD3^-$; representative example in Figure 6C) from healthy donors induced increased levels of apoptosis in both IM-9-JAK1-KO and -JAK2-KO cells when compared with IM-9 cells transduced with an irrelevant shRNA (Figure 6D).

Different target cell lines are more susceptible to NK lysis after silencing of JAK1 or JAK2. Independent experiments conducted with IM-9 cells consistently demonstrated that reduced expression of JAK1 and JAK2 enhanced target cell susceptibility to NK cells. To determine whether inhibition of JAK1 and JAK2 could induce the same effects in other

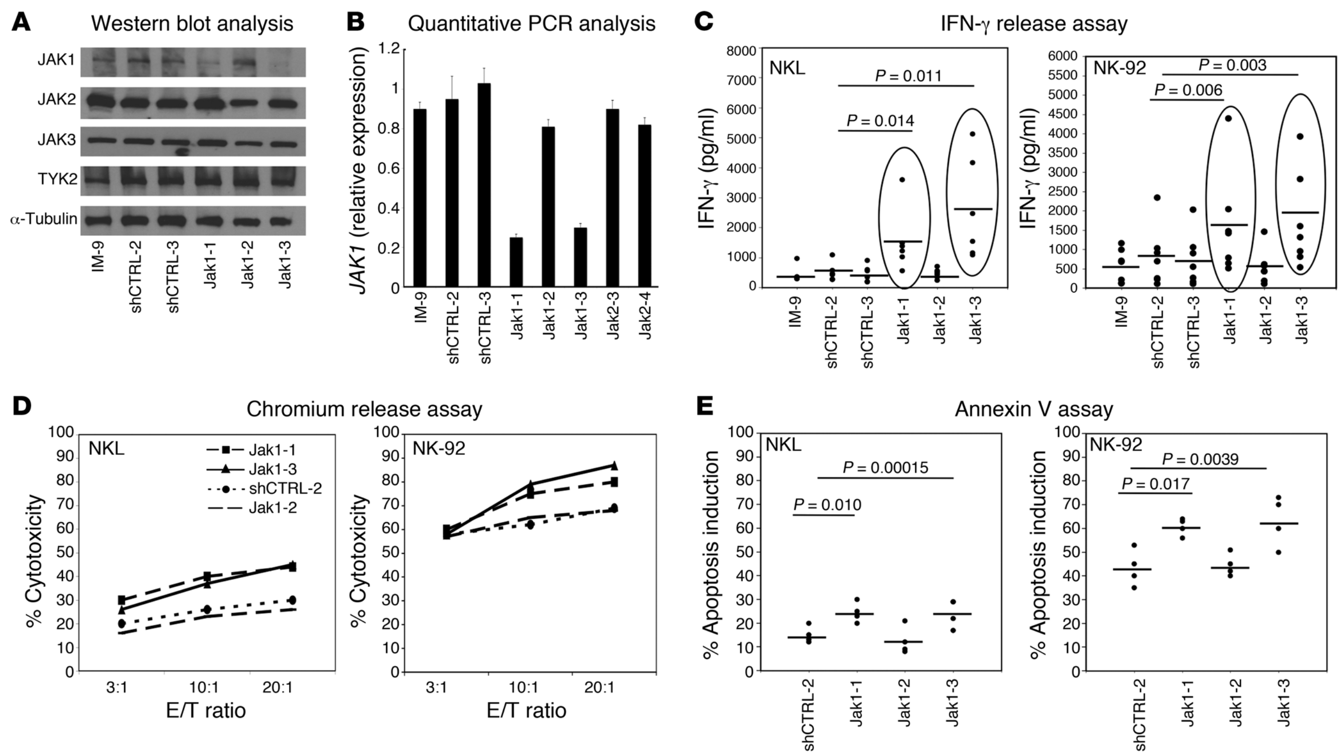


Figure 4

Analysis of IM-9 cells expressing shRNAs targeting JAK1. (**A**) Western blot analysis of IM-9 target cells with stable incorporation of different shRNAs targeting JAK1 (Jak1-1, Jak1-2, and Jak1-3), irrelevant shRNAs (shCTRL-2 and shCTRL-3), and parental IM-9. (**B**) RNAs from parental IM-9 and each IM-9 with stable expression of JAK1, JAK2, and control shRNAs were also evaluated for *JAK1* gene expression. Data represent the relative expression of *JAK1* in parental IM-9 cells, 2 controls (shCTRL-2 and shCTRL-3), IM-9 cells expressing 3 shRNAs targeting JAK1 (Jak1-1, Jak1-2, and Jak1-3), and IM-9 cells expressing 2 shRNAs targeting JAK2 (Jak2-3 and Jak2-4). (**C**) IFN-γ secretion by NKL or NK-92 effector cells incubated with stable IM-9-JAK1-kockdown cells at a 1:1 E/T ratio for 12 hours. Data represent the median of 6 independent experiments with each target cell tested in duplicate. (**D**) Specific lysis of stable IM-9-JAK1-knockdown target cells incubated with NKL or NK-92 effector cells. Percent lysis was determined in a 4-hour chromium release assay for samples incubated at different E/T ratios. Data represent percent killing in 3 different experiments tested in triplicate. (**E**) Percent apoptosis induced by NKL or NK-92 effector cells incubated at a 1:1 E/T ratio for 12 hours with stable IM-9-JAK1-knockdown cells. Cells were stained with a PE-conjugated NKG2A antibody, and the analysis of apoptotic cells was performed on the gated target cells (NKG2A negative). Data represent the mean percent apoptosis induction in 4 independent experiments tested in duplicate. The level of spontaneous apoptosis in IM-9-JAK1-knockdown was subtracted in every experiment.

tumor cells, we infected 2 additional myeloma (KM12BM and INA-6), 3 acute myeloid leukemia (AML) (ML2, U937, and KG-1), 1 chronic myeloid leukemia (K562), and 1 acute T cell leukemia (Jurkat) cell lines with JAK1- and JAK2-specific shRNAs and control shRNA (shCTRL-2). Transduced cells were selected with puromycin to eliminate nontransduced cells, and all cell lines were tested for expression of JAK1 and JAK2. As shown in Figure 7, A and B, target cells with reduced expression of JAK1 or JAK2 induced increased IFN-γ secretion by both NKL and NK-92 effector cells when compared with the same cell lines infected with control shRNA or shRNAs (Jak1-2 and Jak2-1) that did not decrease the protein levels. These experiments confirmed that JAK1 or JAK2 silencing can induce increased susceptibility of multiple types of tumor cells to NK cell activity. However, this effect was higher in myeloma and AML cells (IM-9, KMS12BM, INA6, U937, KG1, ML2) compared with K562 and Jurkat cells, where the increased level IFN-γ secretion by NKL or NK-92 cells in response to JAK1 or JAK2 silencing was less pronounced.

Effects of JAK inhibitors on susceptibility to NK cell–mediated lysis. To determine whether other approaches for targeting JAK1 and JAK2 would also sensitize tumor cells to NK cell activity, we treated 3 cell lines (IM-9, KM12BM, and K562) with 2 different JAK inhibitors (JAK inhibitor 1 and AG-490) at different concentrations. The cytolytic effect of NK cells was assessed by measuring apoptosis of target cells by staining the cultured cells with Annexin V/7AAD and an NK cell marker (NKG2A) to distinguish NK cells from the target cells. Target cells treated with the same concentration of inhibitors but without NK cells were used to determine the level of spontaneous apoptosis induced by the inhibitors (Figure 8A). In each case, incubation with JAK inhibitor alone at these concentrations did not induce apoptosis of the target cells. As shown in Figure 8B, IM-9 cells treated with 10 nM, 30 nM, and 40 nM of JAK inhibitor 1 and subsequently incubated with NK-92 cells resulted in 22.3%, 23.7%, and 27.4% higher levels of apoptosis, respectively, when compared with untreated cells. Similarly, treatment with 0.25 μM, 0.5 μM, and 1 μM of AG-490 and subsequent incubation with NK-92 cells induced 27.7%, 26.7%, and 34% more apoptosis than with untreated cells. Similar effects were also achieved when 2 other target cell lines (KMS12BM and K562) were treated with the same inhibitors. To determine whether this effect was specifically related to inhibition of JAK proteins, we tested IM-9 cells that

Figure 5
Analysis of IM-9 cells expressing shRNAs targeting *JAK2*. (**A**) Western blot analysis of parental IM-9 cells and of IM-9 target cells with stable incorporation of 3 different shRNAs targeting the *JAK2* gene (Jak2-1, Jak2-3, and Jak2-4) and control shRNAs (shCTRL-2 and shCTRL-3). (**B**) RNA from parental IM-9 cells and each IM-9 with stable expression of JAK2, JAK1, and control shRNAs were also evaluated for *JAK2* gene expression. Data represent the relative expression of *JAK2* in IM-9-Jak2-3 and IM-9-Jak2-4 cells compared with *JAK2* expression in IM-9 parental cells, 2 irrelevant controls (shCTRL-2 and shCTRL-3), and IM-9 cells in which JAK1 was silenced with Jak1-1, Jak1-2, and Jak1-3. (**C**) Level of IFN-γ secretion by NKL or NK-92 effector cells incubated with stable IM-9-JAK2-knockdown cells at a 1:1 E/T ratio for 12 hours. Data represent the median of 6 independent experiments, with each target cell tested in duplicate. (**D**) Four-hour chromium release assay measuring specific lysis of IM-9-JAK2-knockdown target cells incubated with NKL or NK-92 cells at different E/T ratios. Data represent the mean percentage of killing in 3 different experiments tested in triplicate. (**E**) Percent apoptosis induced by NKL or NK-92 effector cells incubated with stable IM-9-JAK2-knockdown cells at a 1:1 E/T ratio for 12 hours. Cells were stained with a PE-conjugated NKG2A antibody, and the analysis of apoptotic cells was performed on the gated target cells (NKG2A negative). Data represent the mean percent apoptosis induction in 4 independent experiments tested in duplicate. The level of spontaneous apoptosis in IM-9-JAK2-knockdown cells was subtracted in every experiment.

expressed specific JAK1 and JAK2 shRNAs. As shown in Figure 8C, incubation of IM-9 cells expressing JAK1 and JAK2 shRNAs with NK-92 cells induced 23.5% and 26.4% more apoptosis than incubation with IM-9 cells expressing control shRNAs. This confirmed previous experiments demonstrating that IM-9 cells with reduced expression of JAK1 and JAK2 are more susceptible to NK cell–mediated lysis than controls. However, the level of apoptosis did not increase when IM-9 cells expressing JAK1- and JAK2-targeting shRNAs were treated with either of the JAK inhibitors. These results were also confirmed with purified primary human NK cells (Figure 8E). In contrast, pre-treatment of NKL or NK-92 cells with JAK inhibitor 1 or JAK2 inhibitor did not affect their function and ability to induce apoptosis of IM-9 cells (Figure 8D). These findings indicate that increased sensitivity of target cells to NK-induced apoptosis was specifically related to the level of JAK1 or JAK2 expressed in the target cells.

The effects of JAK inhibitors were also examined in primary tumor cells from 14 patients with hematologic malignancies. This included samples from 4 patients with MM, 5 with AML, and 5 with acute lymphoblastic leukemia (ALL). All samples contained more than 80% blasts (AML and ALL) or CD138⁺ cells (MM). Tumor cells were treated with 3 concentrations of JAK inhibitor 1 for 12 hours and subsequently incubated with NK-92 effector cells at a 1:1 E/T ratio. As shown in Figure 9, MM cells treated with JAK inhibitor were significantly more susceptible to apoptosis induced by NK effector cells. The level of apoptosis at each concentration of JAK inhibitor (10 nM, 30 nM, and 40 nM) was increased by 46.6%, 51%, and 53%, respectively, compared with MM tumor cells incubated in medium alone ($P = 0.0036$, $P = 0.0011$, and $P = 0.0010$, respectively). AML and ALL cells were more susceptible to apoptosis induced by NK-92 cells, and incu-

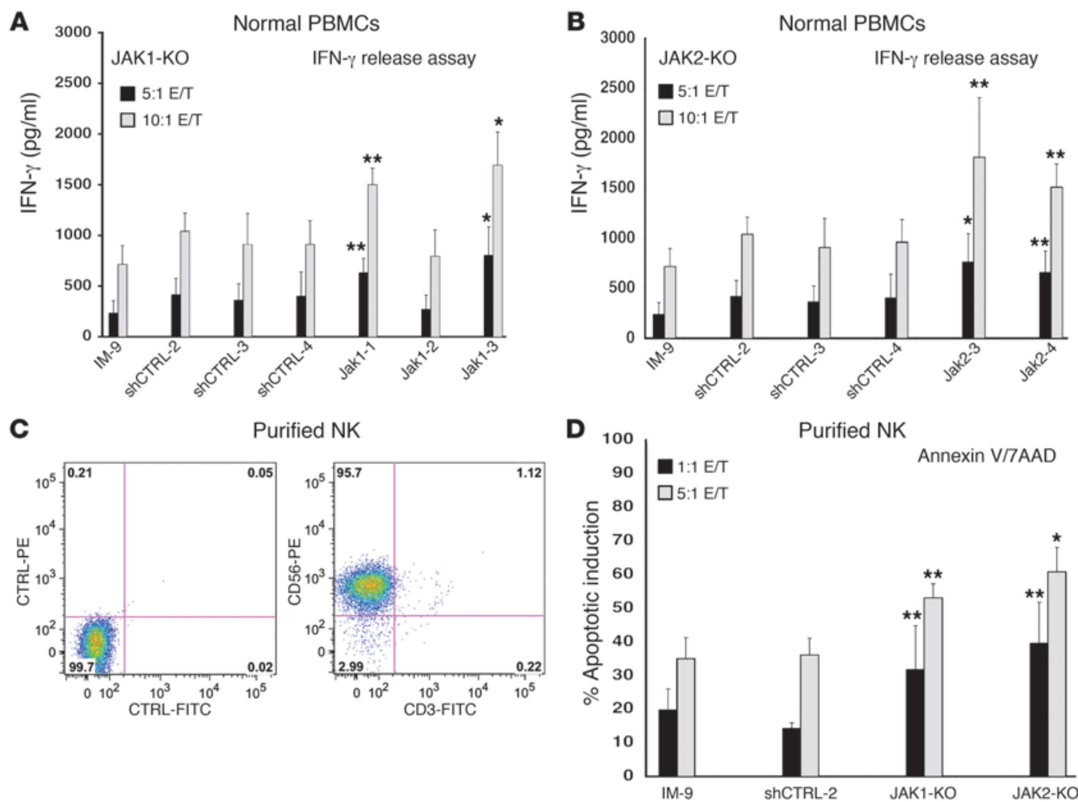


Figure 6

Interaction of IM-9 target cells with primary human NK cells. (**A**) IM-9 cells transfected with Jak1-1, Jak1-2, and Jak1-3 shRNAs or (**B**) Jak2-3 and Jak2-4 shRNAs were incubated with PBMCs from 4 different healthy donors at 5:1 and 10:1 E/T ratios in an IFN-γ release assay. Parental cells and IM-9 cells expressing 3 different shRNAs (shCTRL-2, shCTRL-3, and shCTRL-4) were used as controls in all experiments. In 4 additional experiments, NK cells were purified from PBMCs before incubation with IM-9-JAK1-KO or IM-9-JAK2-KO target cells. (**C**) NK purity (% $CD56^+CD3^-$) was at least 90%. (**D**) Apoptosis of target cells was measured by staining for Annexin V/7AAD using flow cytometry. NK cells were tested at 1:1 and 5:1 E/T ratios. *$P < 0.01$, **$P < 0.05$ compared with shCTRL-2.

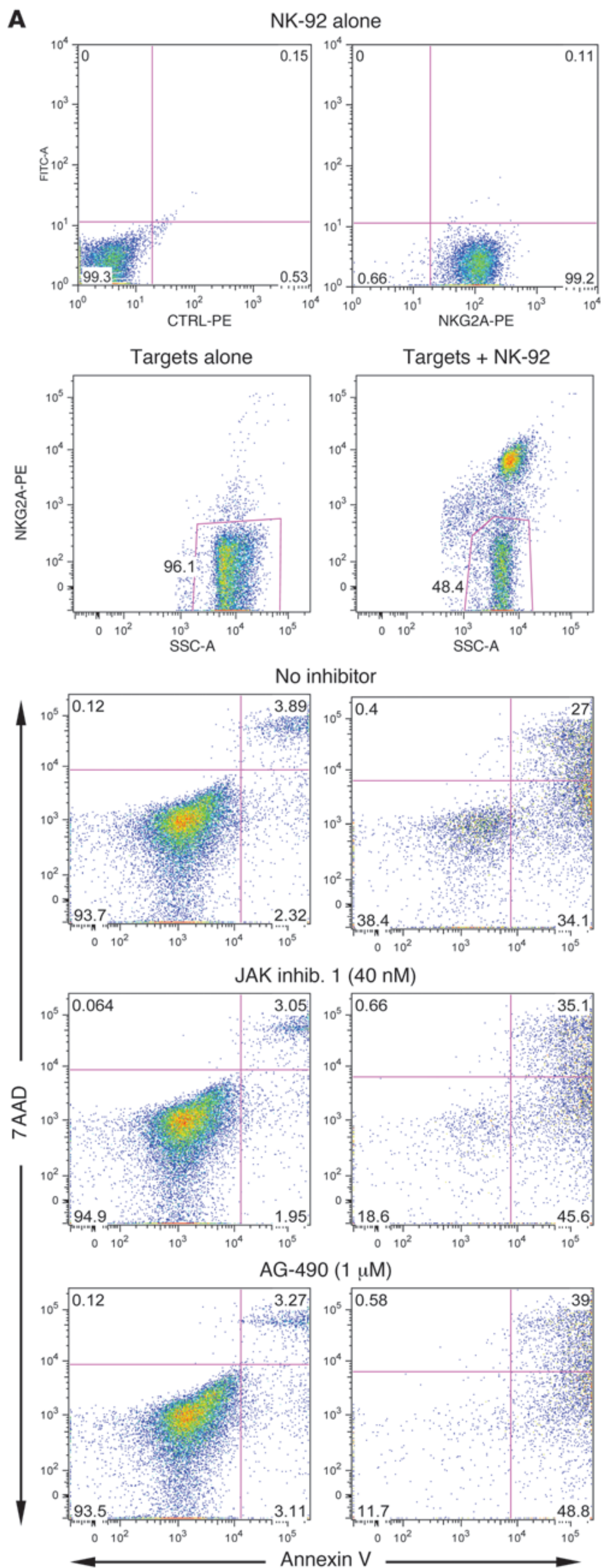
bation of these primary acute leukemia cells with JAK inhibitor also resulted in significantly increased apoptosis. At each concentration of inhibitor, AML apoptosis was increased by 22%, 23%, and 24.5% and ALL apoptosis was increased by 20%, 23.9%, and 21.2%, respectively. Without addition of NK-92 effector cells, apoptosis was less than 9%.

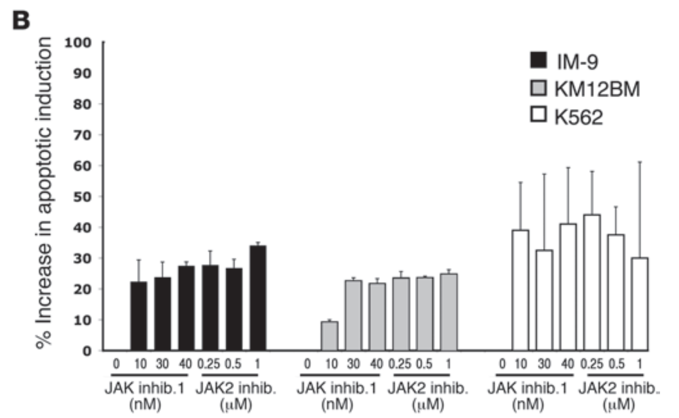
Effects of JAK1 silencing on target cell gene expression. To investigate the mechanisms responsible for increased susceptibility of target cells to NK cell lysis when the *JAK1* gene is knocked down, we utilized gene expression microarrays to compare IM-9-JAK1-KO cells with IM-9 parental cells and IM-9 cells infected with an irrelevant shRNA. Thirty-four genes were found to be highly differentially expressed after JAK1 silencing. As shown in Figure 10A, 13 genes were upregulated and 21 genes were downregulated. JAK1 was the top-scoring downregulated gene, confirming the specificity of the JAK1-targeting shRNAs. Notably, none of the common activating or inhibitory NK cell ligands known to play a role in modulating NK cell activity was found to be differentially expressed in these cells (Supplemental Table 3). Similar expression levels for these ligands were confirmed at the protein level using flow cytometry comparing JAK1-KO cells and JAK2-KO cells with control IM-9 cells transduced with an irrelevant shRNA (Supplemental Figure 4). Interestingly, *TNFRSF10A* (TRAIL-R1) and *CXCL10* were found to be highly upregulated in JAK1-KO cells. Both TRAIL-R1 and CXCL10 have been shown to play important roles in NK cell recognition and activation (22–24). Increased expression of TRAIL-R1 was confirmed by flow cytometry on both JAK1-KO and JAK2-KO cells (Figure 10C). Measurement of CXCL10 by ELISA confirmed increased levels of CXCL10 in JAK1-KO and JAK2-KO supernatants when compared with IM-9 control cells transduced with an irrelevant shRNA (Figure 10B). To better define the relevance of CXCL10 and TRAIL-R1 in the increased sensitivity of JAK1 and JAK2-KO tumor cells to NK cell activity, we co-incubated knockdown cells and irrelevant controls with NKL cells with or without blocking antibodies against CXCL10 and TRAIL-R1. As shown in Figure 10, D and E, in both cases reactivity of NKL cells was reduced in the presence of blocking antibodies. However, while CXCL10 antibodies significantly blocked only the reactivity against JAK1-KO and JAK2-KO lines ($P = 0.04$ and $P = 0.03$, respectively), TRAIL-R1 blocked the reactivity against JAK1-KO, JAK2-KO, as well as the irrelevant controls ($P = 0.003$, $P = 0.0037$, and $P = 0.007$, respectively). Similar results were obtained when NK-92 effector cells were used. Although more experiments will be necessary to completely clarify the mechanisms, these finding suggest that the increased susceptibility of JAK1-KO and JAK2-KO cells could be mostly related to factors secreted by target cells rather than upregulation of activating ligands.

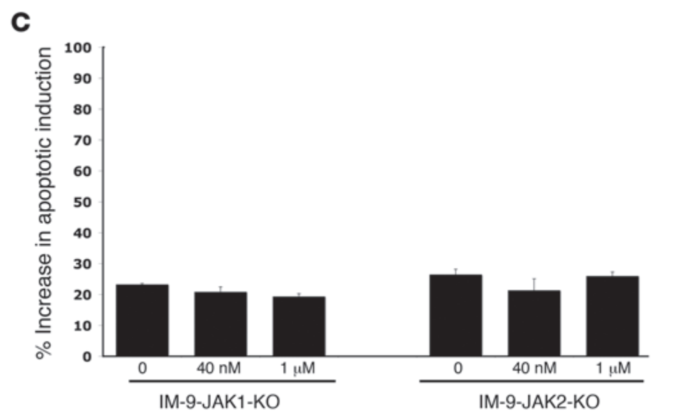
Figure 7
Effects of JAK1 and JAK2 silencing in different leukemia/lymphoma cell lines. Seven tumor cell lines incorporating 3 different shRNAs targeting JAK1 (**A**) and JAK2 (**B**) were tested at a 1:1 E/T ratio with NKL or NK-92 effector cells. Values represent the percent increase in IFN-γ secretion by NK effector cells after incubation with target cells expressing specific shRNAs compared with cells expressing a control shRNA.

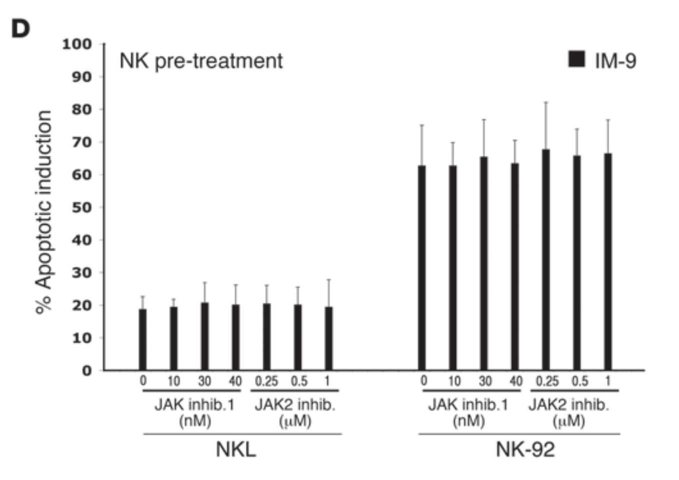
Discussion

Unlike T and B cells of the adaptive immune system, NK cells do not express clonal recognition receptors and do not recognize unique target antigens. Nevertheless, these cells play an important role in immune surveillance and coordinating responses of other immune cells. Most tumor cells express surface molecules that can be recognized by activating receptors on NK cells (25). The expression of these receptors make such cells susceptible to endogenous NK cells, but malignant cells have developed mechanisms to evade innate immune surveillance (26–28). The goal of our studies was to begin to characterize these resistance mechanisms in a broad and unbiased approach. To accomplish this goal, we designed a high-throughput genetic screen to assess interactions between tumor cell targets and NK effector cells. In this assay, tumor cell targets were first transduced with individual lentiviral shRNAs. After integration of shRNAs, NKL effector cells were added to each well, and the interaction between genetically modified target cells and NK effector cells was assessed by measurement of IFN-γ release into the cell culture supernatant. Since our goal was to identify genes that, when silenced, would increase susceptibility to NK cell–mediated lysis, assay conditions were optimized to identify shRNAs that resulted in increased IFN-γ secretion. The lentiviral library we used was a subset of the TRC library that targeted 1,028 genes, including more than 88% of the known human protein kinases and phosphatases (29). We initially focused on protein kinases and phosphatases, since these genes are involved in many cellular functions and their deregulated activity occurs frequently in cancer, where this class of proteins regulates many aspects of cell growth,


A
NK-92 alone
FITC-A
CTRL-PE
NKG2A-PE
0
0.15
99.3
0.53
0.11
0.66
99.2
Targets alone
Targets + NK-92
NKG2A-PE
SSC-A
96.1
48.4
No inhibitor
0.12
3.89
93.7
2.32
0.4
27
38.4
34.1
JAK inhib. 1 (40 nM)
0.064
3.05
94.9
1.95
0.66
35.1
18.6
45.6
AG-490 (1 μM)
0.12
3.27
93.5
3.11
0.58
39
11.7
48.8
7 AAD
Annexin V


B
% Increase in apoptotic induction
IM-9
KM12BM
K562
0 10 30 40 0.25 0.5 1
JAK inhib.1 (nM)
JAK2 inhib. (μM)


C
% Increase in apoptotic induction
0
40 nM
1 μM
IM-9-JAK1-KO
IM-9-JAK2-KO


D
NK pre-treatment
IM-9
% Apoptotic induction
0 10 30 40 0.25 0.5 1
JAK inhib.1 (nM)
JAK2 inhib. (μM)
NKL
NK-92

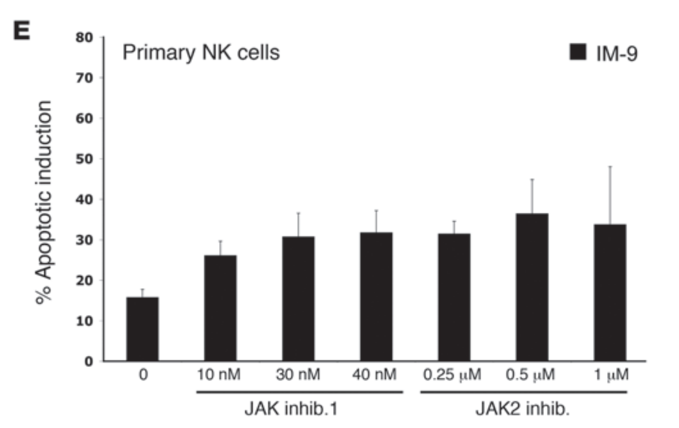

E
Primary NK cells
IM-9
% Apoptotic induction
0
10 nM
30 nM
40 nM
0.25 μM
0.5 μM
1 μM
JAK inhib.1
JAK2 inhib.

Figure 8
Induction of target cell apoptosis by NK cells after treatment of target cells with JAK inhibitors. (**A**) IM-9 target cells treated with JAK inhibitors (JAK inhib.) for 12 hours and subsequently incubated with NK-92 at a 1:1 E/T ratio. NK-92 cells are nearly 100% NKG2A$^+$, and the analysis of apoptotic cells was performed on gated target cells (NKG2A negative). Target cells alone incubated with inhibitors (left scatter plots) were analyzed for the level of apoptosis induced by the JAK inhibitors, and these values were subtracted from the level of apoptosis induced by addition of NK-92 cells (right scatter plots). (**B**) Increased apoptosis induced by NK-92 cells after treatment of target cells with 2 JAK inhibitors. Results are compared with untreated targets incubated with NK-92 cells. Data represent the mean percentages ± SEM obtained in 3 separate experiments. (**C**) IM-9-JAK1-KO and IM-9-JAK2-KO cells were treated with medium alone or 40 nM JAK inhibitor 1 or 1 μM AG-490 for 12 hours and subsequently incubated with NK-92 effector cells. Induced apoptosis was compared with IM-9 cells expressing a control shRNA (shCTRL-2). Data represent the mean ± SEM of 2 separate experiments. (**D**) NKL and NK-92 cells were pretreated with different concentrations of JAK inhibitors and tested for their reactivity against IM-9 using Annexin V/7AAD. Data represent the mean percentages of 3 separate experiments ± SEM tested in duplicate. (**E**) Percent apoptosis induction by purified primary NK cells after IM-9 treatment with different concentrations of 2 JAK inhibitors. *$P < 0.01$, **$P < 0.05$ compared with target cells without inhibitor.

differentiation, adhesion, and death. Interestingly, 79% of the 83 genes that modulated tumor susceptibility to NK activity were protein kinases, while only 4.8% were phosphatases, suggesting a predominant role of protein kinases rather than phosphatases in possible mechanisms of tumor resistance. While several studies have shown that kinases play important roles in immune cell activation (30, 31), no previous studies have suggested that these genes also play a central role in modulating tumor cell susceptibility to elimination by immune cells. Our library also contained shRNAs targeting 372 non-protein kinases, and 12 of the 83 selected genes (14.4%) belonged to this category, suggesting that future studies using a whole genome-wide screening approach could identify many other proteins involved in tumor susceptibility to innate immune surveillance.

Our screening approach was based on the ability of shRNAs to silence the expression of individual genes in tumor cell targets. To avoid off-target effects, the shRNAs included in the TRC library were designed to contain at least 3 mismatches to all known cDNAs in the human genome. We further limited the influence of off-target effects by applying strict selection criteria (11, 16). Among the genes that scored in the top 5th percentile, we only selected genes that induced increased IFN-γ secretion by NKL cells when silenced by at least 2 independent shRNAs, with the second shRNA scoring within the top 20th percentile. Although we employed relatively strict criteria, our approach identified a large set of 83 genes that appear to modulate target cell susceptibility to NK cells. Notably, many of these genes represent common membrane and intracellular signaling pathways that are often activated in malignant cells. For example, 15 of the 83 genes are connected to the MAPK pathway. This pathway has been shown to be involved in many cellular functions, including cell proliferation, cell cycle regulation, cell survival, angiogenesis, and cell migration and is often activated in response to cytokines and growth factors (12, 13). Our screen also identified several membrane receptors such as IGF1R and INSR that can signal through the MAPK and the PIK3 pathways (20). Taken together, these results suggest that many genes can play an important role in tumor susceptibility to immune surveillance and tumor cells can engage multiple pathways and mechanisms to prevent recognition and destruction by endogenous NK cells in vivo.

Independent experiments conducted with stable cell lines incorporating individual shRNAs targeting 5 different genes identified in our screen (*MAPK1*, *IGF1R*, *INSR*, *JAK1*, and *JAK2*) confirmed that increased IFN-γ secretion by NKL cells was specifically associated with reduced expression of the gene in IM-9 target cells. Moreover, this association was also observed with different tumor cell targets and an additional NK effector cell, NK-92, as well as primary NK cells. Finally, increased susceptibility could be measured by increased lysis of target cells as well as by increased secretion of IFN-γ. Overall, 14 of 15 different shRNAs targeting 5 different genes were validated, and only 1 shRNA (INSR-4) was found to induce increased secretion of IFN-γ by NKL cells without measurable downregulation of protein expression. These results support the overall design of our genetic screen and the strict criteria we established to identify genes with functional activity in our assay.

Additional studies focused on JAK family genes, since 2 of the 4 members of this family (JAK1 and JAK2) were identified in our screen. This family of kinases has been functionally well characterized and is known to be associated with cell surface receptors for growth factors, cytokines, chemokines, and immune modulators (32, 33). These kinases play a critical role in cell growth, survival, and development, and activating mutations have been associated with malignant transformation (34, 35). These genes have not previously been associated with tumor cell susceptibility, but because of their importance in many pathways, several specific inhibitors of JAK activity have been developed. For example, a JAK3 inhibitor (CP-690 550) has been found to have immune-suppressive activity in organ transplantation models (36), and clinical trials are underway to test its efficacy in rheumatoid arthritis, psoriasis, and renal transplant rejection. JAK2 inhibitors have potent antitumor activity in solid tumor models (37) and can induce apoptosis of acute lymphoid leukemia and AML cells (38) in combination with other agents. In our studies, we found that silencing of *JAK1* and *JAK2* genes increased tumor cell susceptibility to NK cells but silencing the other 2 members of this family (*JAK3* and *TYK2*) did not have any effect. These results were confirmed in independent experiments where 3 of 4 JAK3 shRNAs and 2 of 4 TYK2 shRNAs selectively downregulated specific protein expression but had no effect on target cell susceptibility to either NKL or NK-92 effector cells. In contrast, silencing of either JAK1 or JAK2 enhanced susceptibility of various tumor cell lines, demonstrating for the first time to our knowledge that these proteins play an important role in tumor cell susceptibility to NK cell lysis. Gene expression profiling experiments showed increased expression of TRAIL-R1 and CXCL10 in IM-9-JAK1-KO cells. However, many known inhibitory/activating ligands such as HLA class I, HLA-A, HLA-C, NKG2D or NCR ligands, CD48 (2B4 ligand), CD155 (DNAM-1 ligand), CD112 (PVR ligand), CD95 (FAS ligand), and adhesion molecules important for cell-cell interactions such as ICAM-1, VCAM-1, CD49d, CD49b and CD49e were not modulated by JAK1 silencing. TRAIL-R1 and CXCL10 have been associated with NK cell recognition and activation (22–24), and their overexpression was confirmed in JAK2-KO as well as JAK1-KO cells. Blocking experiments showed that while CXCL10 antibodies significantly blocked only the reactivity against JAK1- and JAK2-KO lines, TRAIL-R1 equally blocked the reactiv-

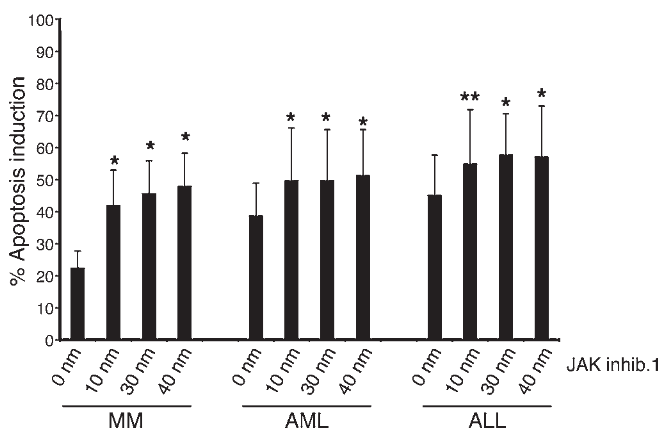


Figure 9
Induction of apoptosis in primary tumor cells by NK cells after treatment with JAK inhibitor. Percent apoptosis induction by NK-92 effector cells after treatment of primary MM (n = 4), AML (n = 5), and ALL (n = 5) tumor cells with different concentration of JAK inhibitor 1. Treated tumor cells were incubated at 1:1 E/T ratio, and the analysis of apoptotic cells was performed on gated target cells (NKG2A negative) using Annexin V/7AAD. *$P < 0.01$, **$P < 0.02$ compared with target cells treated without inhibitor.

ity against JAK1-KO, JAK2-KO, as well as irrelevant controls. These findings suggest that the increased susceptibility of JAK1-KO and JAK2-KO cells could be mostly related to factors secreted by target cells rather than upregulation of activating ligands. CXCL10 antibodies did not completely block the reactivity to the level of the control lines, suggesting that other factors may still contribute to the mechanism. Further experiments will be necessary to gain an understanding of how and whether other molecules are related to the mechanism whereby JAK1 and JAK2 regulate the susceptibility of tumor cells to killing by human NK cells.

To establish the activity of JAK1 and JAK2 as modulators of susceptibility to NK cell lysis, we also tested 2 small molecule inhibitors of JAK1 and JAK2 kinase activity. These studies confirmed that inhibition of these genes in various target cells enhances their susceptibility to apoptosis induced by NK cells. This included primary tumor cells from patients with MM, AML, and ALL, as well as tumor cell lines. This effect of JAK inhibitors was mediated entirely through their inhibition of JAK1 and JAK2 signaling, since they had no effect in tumor cell lines that had already been silenced for these genes. Previous studies have shown that various kinase inhibitors such as dasatinib, which targets SFK and Abl, can also suppress T and NK functions in vivo, suggesting that they could be used as immunomodulatory drugs in autoimmune diseases when administered at higher doses (39, 40). In contrast, kinase inhibitors approved for treatment of renal cell carcinoma such as sorafenib and sunitinib (41, 42) showed differential effects on immune cells activity, especially NK cells (43–45). Although the JAK inhibitors we used in our experiments did not influence the function of NK cells in vitro, the choice and dose of inhibitors used for antitumor treatment should be carefully evaluated when they are combined with immunotherapeutic approaches in patients with cancer.

Taken together, our studies have identified a large set of genes representing several common signaling pathways that appear to modulate tumor cell susceptibility to human NK cells. The unexpected functional role of these genes was uncovered in an unbiased genetic screen, suggesting that many signaling pathways can be utilized by tumor cells to escape immune surveillance. Importantly, many of these pathways are also being targeted by specific inhibitors for potential use as therapeutic agents. Our studies suggest that targeting specific members of these pathways may also enhance the susceptibility of such agents to immune destruction in vivo and this additional activity may enhance the antitumor efficacy of these new therapies.

Methods

High-throughput genetic screen to assess NK cell–target cell interactions

A series of human tumor cell lines were first tested to assess the efficiency of their transduction by lentivirus-based vectors and their maintenance of viability after transduction. IM-9, an MM cell line, was found to have high transduction efficiency under our screening conditions. NKL, a human NK cell line established in our laboratory, was used as a highly reliable source of NK effector cells. NKL cells were derived from a patient with $CD3^-$ $CD56^+$ large granular lymphocyte leukemia and exhibit the morphology of normal activated NK cells. NKL cell growth in vitro is IL-2 dependent, and these cells mediate natural killing as well as IFN-γ secretion when they interact with susceptible target cells in vitro (10).

The genetic screen was performed in a 384-well format using the kinase/phosphatase subset of the TRC shRNA library. This subset contains 476 protein kinases and 180 phosphatases that represent 88% and 80%, respectively, of known NCBI sequences with these functions (29). The library also includes 372 genes representing tumor suppressors, DNA binding proteins, and modification enzymes, as previously described (11). Each gene is targeted by an average of 5 distinct shRNAs. As shown in the schema in Figure 1A, 2,000 IM-9 myeloma cells/well were plated in 384-well plates in 5 replicate sets, and each set was transduced with the same individual shRNA-expressing vectors. After 24 hours incubation at 37°C, the medium was changed, and puromycin (2 μg/ml) was added to one set. Forty-eight hours after puromycin selection, cell viability (CellTiter-Glo, Promega) was determined in 2 of the replicate sets, one treated with puromycin and one left untreated after transduction to assess both infection efficiency and potential toxicity of each shRNA. Six thousand NKL effector cells were added to each well in the remaining 3 sets. After 12 hours incubation at 37°C, individual supernatants were harvested and transferred to 96-well format plates. The concentration of IFN-γ in each supernatant was measured in 2 replicate sets (12,288 wells) using human CBA IFN-γ Flex Set capture beads according to the manufacturer's protocol (BD Biosciences). One replicate set of harvested supernatants was kept as a backup. CBA IFN-γ beads were analyzed using a BD FACSCanto II flow cytometer equipped with a high-throughput platform and results analyzed using FCAP Array software (Soft Flow Inc). All steps were performed using μFill (BioTek U.S.) and Tecan robotic stations to ensure reproducibility.

Generation of stable shRNA-expressing cell lines

Glycerol stocks containing pLKO.1 lentiviral vectors of interest were obtained from TRC. Each pLKO.1 plasmid containing a specific shRNA was prepared from glycerol stocks and transfected together with pMD-VsVg and

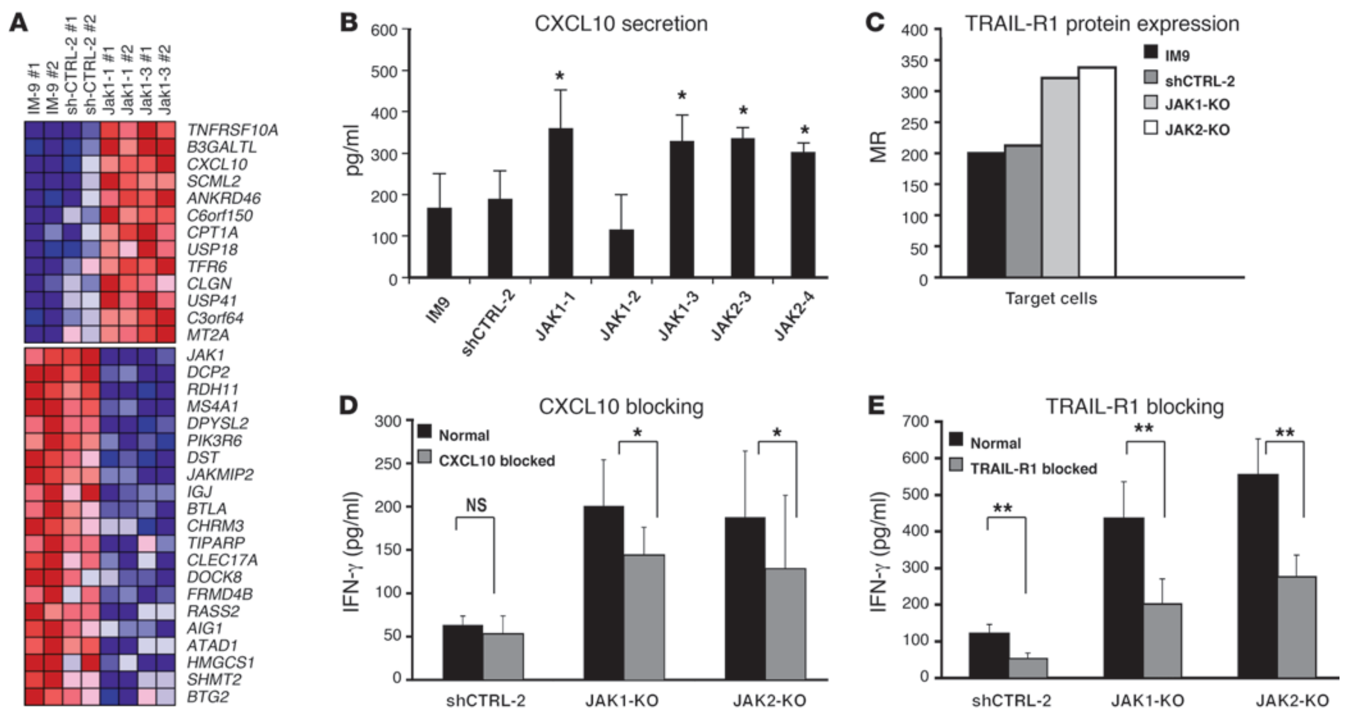


Figure 10

Top 34 differentially expressed genes in IM-9-JAK1-KO compared with control IM-9 cells. (**A**) Gene expression of 2 independent experiments (#1 and #2) using the Gene 1.0 ST Array. Samples from IM-9 cell lines expressing 2 JAK1 shRNAs (Jak1-1 and Jak1-3) were compared with IM-9 cells expressing an irrelevant shRNA (shCTRL-2) and with IM-9 parental cells. Top-scoring genes were defined by a minimal fold-change of 1.5 and maximal *q* value of 0.4. (**B**) IM-9-JAK1, JAK2-KO, and control cells were cultured for 12 hours and CXCL10 in culture supernatant was measured using an ELISA assay. Data represent the mean values ± SEM of 3 independent experiments run in triplicate. *$P < 0.05$ compared with IM-9 cells transduced with an irrelevant shRNA. (**C**) IM-9-JAK1-KO, IM-9-JAK2-KO, IM-9, and IM-9 cells transduced with an irrelevant shRNA (shCTRL-2) were stained with an anti–TRAIL-R1–PE antibody and analyzed by flow cytometry. (**D**) IM-9-JAK1-KO, IM-9-JAK2-KO, and IM-9-shCTRL-2 cells were co-incubated with NKL at a 1:1 E/T ratio with or without CXCL10 blocking antibody for 12 hours. Data represent the mean values + SEM of IFN-γ secreted by NKL cells in 4 independent experiments. (**E**) IM-9-JAK1-KO, IM-9-JAK2-KO, and IM-9-shCTRL-2 cells were co-incubated with NKL at a 1:1 E/T ratio with or without TRAIL-R1–Fc chimera for 12 hours. Data represent the mean values + SEM of IFN-γ secreted by NKL cells in 3 independent experiments. *$P < 0.05$, **$P < 0.01$.

pCMV delta 8.9 in HEK293T packaging cell line to produce virus supernatants using FuGENE (Roche Applied Science). Target cell lines were transduced with virus supernatants and Polybrene at 8 μg/ml (Millipore) two times and selected with puromycin 24 hours after the second transduction.

IFN-γ and cytotoxicity assays

Stable cell lines expressing individual shRNAs were incubated with NKL or NK-92 cells at a 1:1 E/T ratio or primary human PBMCs at 5:1 and 10:1 E/T ratios at 37°C for 12 hours. In several experiments NK cells were purified from PBMCs using the MACS magnetic cell separation system and NK cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec). Isolated NK cells were tested for purity using CD56 and CD3 antibodies; NK cell purity was greater than 90% in each experiment. After coculture, supernatants were harvested and incubated with CBA IFN-γ beads according to the manufacturer's instruction (BD Biosciences), and the level of IFN-γ produced by NK effector cells was determined by flow cytometry using a BD FACSCanto II flow cytometer. For IFN-γ intracellular staining, IM-9-JAK1-KO cells were incubated with NKL effector cells for 4 hours in the presence of brefeldin A. Cocultured cells were harvested and stained with anti-CD2–FITC, followed by a fixation/permeabilization step using BD Cytofix/Cytoperm kit (BD Biosciences), and subsequently stained with a PE-conjugated anti–IFN-γ antibody. Staining for IFN-γ was analyzed separately for $CD2^+$ NKL cells and $CD2^-$ tumor cells.

For coculture with CXCL10 and TRAIL-R1 blocking experiments, we co-incubated IM-9-JAK1-KO, JAK2-KO, and IM-9-shCTRL-2 cells with NKL or NK-92 with or without CXCL10 antibodies (1.2 μg/ml) or TRAIL-R1-Fc (1 μg/ml) (R&D Systems) overnight at a 1:1 E/T ratio. Supernatants were harvested 12 hours later and analyzed for IFN-γ concentration using CBA IFN-γ beads as described above.

Cytotoxicity was measured using radiolabeled target cells in a 4-hour ^{51}Cr release assay. Effector cells and target cells were plated at 5,000 cells/well and co-incubated at different E/T ratios: 3:1, 10:1, and 20:1. Spontaneous release was determined by incubating target cells with medium alone, and maximum release was obtained by lysing cells in 10% NP-40. Percent specific cytotoxicity was calculated by the following formula: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Induction of apoptosis by NK cells of JAK1-KO and JAK2-KO cells was determined using flow cytometry. IM-9-JAK1-KO and IM-9-JAK2-KO or control cells were incubated with NKL or NK-92 cells at a 1:1 E/T ratio for 12 hours. Cells were subsequently stained with anti–Annexin V–FITC and anti-NKG2A–PE antibody. The percent apoptotic cells (Annexin V positive) was determined by gating on the target cell population (NKG2A negative). The level of spontaneous apoptosis of target cells incubated with medium alone was subtracted in every experiment.

Measurement of protein and gene expression

Western blot analysis. Cell lines with stable expression of individual shRNAs after puromycin selection were lysed using RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitors (Roche), and PMSF. Lysates were subject to 7.5% SDS-PAGE (Ready Gel, Bio-Rad) with Tris-glycine buffer and transferred onto nitrocellulose membranes in 20% methanol in Tris-glycine buffer. Membranes were stained with rabbit anti-JAK1, -JAK2, -JAK3, -TYK2, -ERK1/2, and -α-tubulin (Cell Signaling Technology), followed by a secondary horseradish peroxidase–conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc.), and visualized by chemiluminescence.

Flow cytometry. Expression of cell surface proteins was assessed by flow cytometry. 5×10^5 cells expressing individual shRNAs and control cells were incubated with mouse anti-IGF1R (CD221) (AbD Serotec) and mouse anti-INSR (CD220) (BD Biosciences — Pharmingen), followed by RPE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.). Modulation of inhibitory/activating ligands on JAK1-KO and JAK2-KO cells was assessed using mouse anti–class I (clone W6/32), anti–HLA-A2 (clone BB7.2), goat anti–HLA-C (Santa Cruz Biotechnology Inc.), human NKp30-Fc, NKp44-Fc, NKp46-Fc, NKG2D-Fc, and CD155 (R&D Systems), followed by RPE-conjugated goat anti-mouse IgG, goat anti-human IgG, and donkey anti-goat IgG (Jackson ImmunoResearch Laboratories Inc.). PE-conjugated anti-CD49d, -CD49b, -CD49e, –ICAM-1, –VCAM-1 were from BD Biosciences — Pharmingen), and PE-conjugated anti–TRAIL-R1, –TRAIL-R2, -CD95, and -CD112 and FITC-conjugated CD48 were from Beckman Coulter/Immunotech. A minimum of 15,000 gated cells were acquired using a BD FACSCanto II flow cytometer, and data were analyzed using FlowJo software (Tree Star Inc.).

Quantitative RT-PCR. RNA was extracted using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions, and 1 μg was used for reverse transcription (QuantiTect kit, QIAGEN). Real-time PCRs were performed on an ABI PRISM 7700 system using SYBR green–based assays with AmpliTaq Gold (Applied Biosystems). All reactions were performed in triplicate. Quantitative gene expression was calculated from the Ct values for each reaction using the average reaction efficiency for each primer pair. Data were normalized to *TBP* and *UBQLN1* (housekeeping genes) and scaled to the mean of the controls to obtain relative expression values. Primer sets for JAK1 and JAK2 were CCCATTCATCAAGCTCAGTGAC/CACACTCAGGTTCTTGGAGTCCT, AAAGGATCTGGTATCCACCCAAC/GATATTCCATGCCGATAGGCTCT.

JAK inhibitor treatment

IM-9, KMS12BM, and K562 cells were treated for 12 hours with 0, 10, 30, and 40 nM JAK inhibitor 1 (Calbiochem, EMD Biosciences) and 0.25, 0.5, and 1 μM JAK2 inhibitor AG-490 (Enzo, Life Sciences). After 12 hours at 37°C, treated cells were washed and incubated with NK-92 cells for an additional 12 hours. Apoptosis induction of target cells was determined by flow cytometry using an Annexin V/7AAD assay. PE-conjugated anti-NKG2A antibody (Beckman Coulter) was used to detect and exclude NK effector cells from the analysis, and the level of apoptosis was only calculated for NKG2A-negative cells. The level of spontaneous apoptosis of target cells without NK cells was subtracted in every experiment.

JAK inhibitor treatment in primary leukemia cells

Primary tumor cells from patients with MM (n = 4), AML (n = 5), and ALL (n = 5) containing at least 80% blasts or CD138⁺ cells were incubated with 0, 10, 30, and 40 nM JAK inhibitor 1 (Calbiochem, EMD Biosciences) for 12 hours and subsequently incubated for 12 hours at a 1:1 E/T ratio with NK-92 cells. AML and ALL samples contained at least 80% blasts, and MM samples contained at least 80% $CD138^+$ cells. Apoptosis induction of target cells by NK-92 cells was determined by flow cytometry using Annexin V/7AAD as described above.

Gene expression profile of JAK1-knockdown cells

Total RNA was isolated from cells lysed in TRIzol (Invitrogen); converted into fragmented, biotinylated cDNA hybridized to GeneChip microarray chips (Gene 1.0 ST); and fluorescently labeled according to the standard protocol (Affymetrix) at the DFCI microarray core facility. Raw data were processed in Expression Console (Affymetrix) using RMA normalization. Expression values for each gene were annotated by mapping all probe sets to human genome version hg19. Data complexity was reduced to one canonical transcript per gene, resulting in a single identifier per gene (17,982 total). The expression data were processed in GenePattern (http://www.broadinstitute.org/cancer/software/genepattern/) (46). Nonexpressed genes were filtered out, and the resulting expression matrix was analyzed with the comparative marker module (2-class comparison, asymptotic P values) in GenePattern. Top-scoring genes were defined by a minimal fold change of 1.5 and maximal q value of 0.4. Data are available in the GEO database under the accession number GSE37012.

Statistics

B-score analysis was performed in 2 replicates treated with and without puromycin (12,288 wells) to determine systematic measurement offsets for each row and column in the plates (14). Normalized B-score values were analyzed to exclude all wells where the viability of infected cells was poor due to virus toxicity or low shRNA infection rate. This analysis reduced the number of evaluable wells for IFN-γ measurements from 6,144 to 4,177. The means of the IFN-γ values obtained from the 2 replicates with NKL cells added were normalized among the 16 library plates using z-score analysis. Two standard deviations above the mean of the 4,177 standardized values ($z = 0.108 \pm 1.09$), equivalent to the top 5th percentile, was set as a cut-off for positive values. Student t test was used for all 2-sample comparisons. A P value less than 0.05 was considered significant.

Study approval

Patient tumor samples were obtained under a protocol approved by the Institutional Review Board of the Dana-Farber/Harvard Cancer Center, and informed consent was obtained from each patient.

Acknowledgments

This work was supported by NIH grants P01 CA078378 (to J. Ritz) and R21 AI088521 (to R. Bellucci), the International Myeloma Foundation (IMF), Multiple Myeloma Research Foundation (MMRF), and Claudia Adams Barr Research Program (to R. Bellucci). We thank Amanda Carmignani for helpful suggestions and discussion on the statistical analysis.

Received for publication July 1, 2011, and accepted in revised form May 2, 2012.

Address correspondence to: Jerome Ritz, Dana-Farber Cancer Institute, 450 Brookline Ave., M530, Boston, Massachusetts 02215, USA. Phone: 617.632.3465; Fax: 617.632.5167; E-mail: Jerome_Ritz@dfci.harvard.edu. Or to: Roberto Bellucci, Dana-Farber Cancer Institute, 450 Brookline Ave., M526, Boston, Massachusetts 02215, USA. Phone: 617.632.3536; Fax: 617.632.5167; E-mail: Roberto_Bellucci@dfci.harvard.edu.

1. Caligiuri MA. Human natural killer cells. *Blood*. 2008;112(3):461–469.
2. Vivier E, et al. Innate or adaptive immunity? The example of natural killer cells. *Science*. 2011; 331(6013):44–49.
3. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol*. 2008;9(5):495–502.
4. Moretta L, Biassoni R, Bottino C, Mingari MC, Moretta A. Human NK-cell receptors. *Immunol Today*. 2000;21(9):420–422.
5. Berns K, et al. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. *Nature*. 2004;428(6981):431–437.
6. Root DE, Hacohen N, Hahn WC, Lander ES, Sabatini DM. Genome-scale loss-of-function screening with a lentiviral RNAi library. *Nat Methods*. 2006;3(9):715–719.
7. Silva JM, et al. Second-generation shRNA libraries covering the mouse and human genomes. *Nat Genet*. 2005;37(11):1281–1288.
8. Firestein R, et al. CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. *Nature*. 2008;455(7212):547–551.
9. Tiedemann RE, et al. Kinome-wide RNAi studies in human multiple myeloma identify vulnerable kinase targets, including a lymphoid-restricted kinase, GRK6. *Blood*. 2010;115(8):1594–1604.
10. Robertson MJ, Cochran KJ, Cameron C, Le JM, Tantravahi R, Ritz J. Characterization of a cell line, NKL, derived from an aggressive human natural killer cell leukemia. *Exp Hematol*. 1996;24(3):406–415.
11. Moffat J, et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell*. 2006;124(6):1283–1298.
12. Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature*. 2001;410(6824):37–40.
13. Schaeffer HJ, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol*. 1999;19(4):2435–2444.
14. Brideau C, Gunter B, Pikounis B, Liaw A. Improved statistical methods for hit selection in high-throughput screening. *J Biomol Screen*. 2003; 8(6):634–647.
15. Jackson AL, Linsley PS. Noise amidst the silence: off-target effects of siRNAs? *Trends Genet*. 2004; 20(11):521–524.
16. Sharma S, Rao A. RNAi screening: tips and techniques. *Nat Immunol*. 2009;10(8):799–804.
17. McCubrey JA, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta*. 2007;1773(8):1263–1284.
18. Nair PN, De Armond DT, Adamo ML, Strodel WE, Freeman JW. Aberrant expression and activation of insulin-like growth factor-1 receptor (IGF-1R) are mediated by an induction of IGF-1R promoter activity and stabilization of IGF-1R mRNA and contributes to growth factor independence and increased survival of the pancreatic cancer cell line MIA PaCa-2. *Oncogene*. 2001;20(57):8203–8214.
19. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. *Leukemia*. 1994;8(4):652–658.
20. Baserga R. The insulin-like growth factor-I receptor as a target for cancer therapy. *Expert Opin Ther Targets*. 2005;9(4):753–768.
21. Shaw RJ, Cantley LC. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature*. 2006;441(7092):424–430.
22. Johnstone RW, Frew AJ, Smyth MJ. The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer*. 2008;8(10):782–798.
23. Shimizu K, Asakura M, Fujii S. Prolonged antitumor NK cell reactivity elicited by CXCL10-expressing dendritic cells licensed by CD40L+ CD4+ memory T cells. *J Immunol*. 2011;186(10):5927–5937.
24. Taub DD, Sayers TJ, Carter CR, Ortaldo JR. Alpha and beta chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J Immunol*. 1995;155(8):3877–3888.
25. Lanier LL. NK cell recognition. *Annu Rev Immunol*. 2005;23:225–274.
26. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002;3(11):991–998.
27. Orr MT, Lanier LL. Natural killer cell education and tolerance. *Cell*. 2010;142(6):847–856.
28. Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol*. 2006;90:1–50.
29. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. 2002;298(5600):1912–1934.
30. Jiang K, et al. Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nat Immunol*. 2000;1(5):419–425.
31. Wei S, et al. Direct tumor lysis by NK cells uses a Ras-independent mitogen-activated protein kinase signal pathway. *J Immunol*. 2000;165(7):3811–3819.
32. Bellido T, Borba VZ, Roberson P, Manolagas SC. Activation of the Janus kinase/STAT (signal transducer and activator of transcription) signal transduction pathway by interleukin-6-type cytokines promotes osteoblast differentiation. *Endocrinology*. 1997;138(9):3666–3676.
33. Nicholson SE, Oates AC, Harpur AG, Ziemiecki A, Wilks AF, Layton JE. Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation. *Proc Natl Acad Sci U S A*. 1994;91(8):2985–2988.
34. Jelinek J, et al. JAK2 mutation 1849G>T is rare in acute leukemias but can be found in CMML, Philadelphia chromosome-negative CML, and megakaryocytic leukemia. *Blood*. 2005;106(10):3370–3373.
35. Lacronique V, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science*. 1997;278(5341):1309–1312.
36. Conklyn M, Andresen C, Changelian P, Kudlacz E. The JAK3 inhibitor CP-690550 selectively reduces NK and CD8+ cell numbers in cynomolgus monkey blood following chronic oral dosing. *J Leukoc Biol*. 2004;76(6):1248–1255.
37. Hedvat M, et al. The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell*. 2009;16(6):487–497.
38. Ferrajoli A, et al. WP1066 disrupts Janus kinase-2 and induces caspase-dependent apoptosis in acute myelogenous leukemia cells. *Cancer Res*. 2007;67(23):11291–11299.
39. Blake SJ, Bruce Lyons A, Fraser CK, Hayball JD, Hughes TP. Dasatinib suppresses in vitro natural killer cell cytotoxicity. *Blood*. 2008;111(8):4415–4416.
40. Schade AE, et al. Dasatinib, a small-molecule protein tyrosine kinase inhibitor, inhibits T-cell activation and proliferation. *Blood*. 2008;111(3):1366–1377.
41. Escudier B, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med*. 2007; 356(2):125–134.
42. Goodman VL, et al. Approval summary: sunitinib for the treatment of imatinib refractory or intolerant gastrointestinal stromal tumors and advanced renal cell carcinoma. *Clin Cancer Res*. 2007;13(5):1367–1373.
43. Hipp MM, et al. Sorafenib, but not sunitinib, affects function of dendritic cells and induction of primary immune responses. *Blood*. 2008;111(12):5610–5620.
44. Krusch M, et al. The kinase inhibitors sunitinib and sorafenib differentially affect NK cell antitumor reactivity in vitro. *J Immunol*. 2009;183(12):8286–8294.
45. Ozao-Choy J, et al. The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies. *Cancer Res*. 2009;69(6):2514–2522.
46. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP. GenePattern 2.0. *Nat Genet*. 2006; 38(5):500–501.