Cellular senescence is an established cellular stress response, primarily acting to limit the proliferative potential of cells (Collado and Serrano, 2010). It can be triggered in many cell types in response to diverse cellular damage (Collado and Serrano, 2010). An important trigger of senescence is oncogenic stress, mediated by activation of p53/p21 and p16/Rb tumor suppressor pathways, which promote senescence by transactivating genes that arrest cell cycle progression and promote the senescent state (Serrano et al., 1997; Narita et al., 2003; Braig et al., 2005; Michaloglou et al., 2005; Ventura et al., 2007). It is believed that senescence is a key mechanism by which p53 suppresses tumorigenesis (Braig and Schmitt, 2006; Collado and Serrano, 2010). The senescent state is associated with several phenotypic alterations, including the secretion of soluble factors involved in the maintenance of the senescent state (e.g., CXCL2 [Acosta et al., 2008], PAI-1 [plasminogen activator inhibitor-1; Kortlever et al., 2006], IGFBP7 [insulin-like growth factor-binding protein 7; Wajapeyee et al., 2008]), and other molecules that regulate the immune response (cytokines and chemokines; Kuilman et al., 2008; Rodier et al., 2009, 2011), angiogenesis (vascular endothelial growth factor), and other processes (Coppé et al., 2006). This so-called senescence-associated secretory phenotype (SASP), as well as the resulting immune responses, could promote or repress cancer progression in a context-dependent manner (Rodier and Campisi, 2011).

With respect to immune responses, the senescent state has similarly been associated with alterations that promote tumorigenesis (Krtolica et al., 2001; Bavik et al., 2006; Yang et al., 2006; Liu and Hornsby, 2007) but in other cases with immune-mediated tumor elimination (Xue et al., 2007; Krizhanovsky et al., 2008; Kang et al., 2011).

Accumulating evidence suggests that immune-mediated destruction of senescent cells

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**CORRESPONDENCE**

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may play a role in tumor surveillance as well as in resolution of fibrotic injury to tissues (Xue et al., 2007; Krizhanovsky et al., 2008; Kang et al., 2011; Lujambio et al., 2013). In some cases, immune cells such as NK cells and other immune effector cells like granulocytes and macrophages have been implicated in mediating these effects (Xue et al., 2007; Krizhanovsky et al., 2008; Lujambio et al., 2013).

NK cells are lymphocytes that kill tumor cells and infected cells and secrete various inflammatory cytokines, including IFN-γ and TNF (Vivier et al., 2011). Like other lymphocytes and immune cells, NK cells are recruited to infected or transformed tissue by the action of chemokine gradients (Grégoire et al., 2007). NK cell killing requires engagement of specific ligands on tumor cells by NK receptors. Some NK receptors, specific for MHC I molecules, inhibit NK activity, whereas other receptors activate NK functions (Vivier et al., 2011). Several activating NK receptors have been implicated in the killing of tumor cells. The best characterized such receptor is NKG2D (encoded by the Klrk1 gene), which is expressed by all NK cells. NKG2D binds to each of 5–10 (depending on the individual) different MHC I–related cell surface ligands, including the RAE-1/MULT1/H60 subfamilies of proteins in mice and the MICA/ULBP subfamilies of proteins in humans (Raulet, 2003). The ligands are expressed poorly by normal cells but are often induced on cancer cells as the result of stress pathways or other pathways that are dysregulated in cancer cells (Raulet et al., 2013). NKG2D has been implicated in immune surveillance of tumors using transgenic models of spontaneous cancer as well as subcutaneous tumor transfer models (Cervera et al., 2001; Diefenbach et al., 2001; Guerra et al., 2008).

A recent paper suggested that senescent tumors are targeted for elimination by NK cells and other innate effector cells (Xue et al., 2007). However, it is unknown how p53-expressing senescent tumors mobilize the natural killer cell response. Nor is it known how NK cells recognize the senescent tumors. In this study, we sought to define how NK cells carry out this function by defining the receptors and ligands involved and the alterations in senescent cells that mobilize the NK cell response. Our results demonstrate that induced expression of p53 in a model of transferred liver tumor cells causes the production of various chemokines, including CCL2, and that CCL2 is essential for robust recruitment of NK cells into the tumor. The NK-depended component of tumor cell elimination is completely dependent on NKG2D-mediated recognition of RAE-1 proteins expressed on the tumor cells, but RAE-1 expression is not induced by p53 expression because it is robust on the tumor cells even before p53 expression is induced. The results suggest that other types of signals associated with the transformed state induce expression of NKG2D ligands, but p53 expression mobilizes effective NK-dependent tumor elimination by inducing CCL2 expression that recruits NK cells into the tumor.

**RESULTS**

**NK- and NKG2D-dependent elimination of senescent tumors**

We used a mouse model of tumor senescence where p53 expression in H-RasV12–transformed liver carcinoma cells (TRE_shp53 cells) is regulated by an inducible p53 shRNA, which can be extinguished in response to doxycycline (Xue et al., 2007). Hence, tumors initiated under conditions of p53 repression can be allowed to progress or can be induced by doxycycline to express p53 and hence undergo senescence in vivo (Xue et al., 2007). Using this system, it was previously reported that when tumors lacking p53 expression were first established in T cell–deficient nude mice, subsequent induction of p53 with doxycycline resulted in the arrest of tumor growth and the gradual disappearance of the senescent tumors.

![Figure 1. NKG2D-dependent elimination of senescent tumors by NK cells. (A) Rag2-/-, Rag2-/- Klrk1-/-, and NK-depleted Rag2-/- mice (n = 6 to 11 per group) were injected subcutaneously with 2 × 10^6 liver tumor cells with inducible p53. 10 d after injection, tumor sizes were measured and doxycycline was added to the drinking water. This day was designated day 0. Tumor sizes were monitored 3, 5, 7, 9, 11, and 13 d later. (B) Relative tumor volumes (day 0 = 1) for each group of mice are shown. Values represent mean ± SEM for each group. Two-way ANOVA and Bonferroni's tests were performed at each time point. *P < 0.05; **P < 0.01; ***P < 0.001.](image)
(Xue et al., 2007). Notably, the elimination of the senescent tumors was prevented or delayed when NK cells, macrophages, or granulocytes were depleted individually from the host animals at the time of doxycycline treatment (Xue et al., 2007).

In our experiments, transformed liver tumor cells were transferred subcutaneously to B6–Rag2\(^{−/−}\) mice, which lack T cells and B cells. p53 induction resulted in the arrest and gradual disappearance of the tumors (Fig. 1). Depletion of NK cells with NK1.1 antibody early before and after inducing p53 resulted in a significant delay in tumor elimination compared with mice treated with control IgG, corroborating the role of NK cells in the elimination of senescent tumors (Fig. 1). The tumors were still eliminated at later time points, suggesting that other mechanisms independently cause tumor elimination. These latter mechanisms may depend on neutrophils and/or macrophages (Xue et al., 2007). In subsequent experiments, we focused on the role of NK cells in the rapid elimination of senescent tumors.

To test the role of NKG2D in elimination of senescent tumors, transformed liver tumor cells were transferred subcutaneously to mice lacking both RAG2 and NKG2D (Rag2\(^{−/−}\)Klrk1\(^{−/−}\)) as well as to control Rag2\(^{−/−}\)Klrk1\(^{+/+}\) mice. After treatment of the mice with doxycycline, senescent tumor elimination was delayed in Rag2\(^{−/−}\)Klrk1\(^{−/−}\) mice, to a similar extent as was observed in mice depleted of NK cells (Fig. 1). A similar delay was observed in Rag2\(^{−/−}\) mice that were injected with NKG2D antibodies to block ligand recognition (Fig. 1 C). Furthermore, these delays were of a similar magnitude as occurred in mice depleted of NK cells, the only known cells to express NKG2D in Rag2\(^{−/−}\) mice. Therefore, these data demonstrate that NKG2D plays an essential role in the rapid elimination of senescent tumors by NK cells.

**p53 restoration is not necessary for expression of NKG2D ligands in the liver tumor cells**

The finding that NKG2D plays a role in eliminating senescent tumors led us to investigate the possibility that p53 expression or the senescent state is associated with the induced expression of NKG2D ligands. Transformed fetal liver cells cultivated in vitro in the p53-off state expressed substantial amounts of one NKG2D ligand, RAE-1\(\alpha\), but did not express MULT1 or H60 family ligands (Fig. 2 A). Cultivation of these cells in vitro with doxycycline for 7 d did not result in a significantly increased expression of any of the NKG2D ligands at either the protein level (as shown by staining with ligand-specific antibodies or by staining with NKG2D-Fc, which binds to all NKG2D ligands; Fig. 2 A) or mRNA level (Fig. 2 C). Induction of p53 in vivo, in established tumors implanted in Rag2\(^{−/−}\)Klrk1\(^{−/−}\) recipients, also did not result in consistent or significantly increased expression of RAE-1\(\alpha\) (Fig. 2, B and D) or other NKG2D ligands (Fig. 2 A and not depicted), as shown by ex vivo analysis of tumor cell suspensions. These data suggest that RAE-1 expression occurs without p53 induction in this model and that p53 restoration and/or the senescent state do not further induce the expression of NKG2D ligands.

Although p53 expression was not associated with increased expression of NKG2D ligands, it remained possible that the induction of senescence rendered the cells more susceptible to NKG2D-dependent lysis. As shown in Fig. 3 A, however, p53-restoration and the accompanying senescence did not increase the sensitivity of the tumor cells to killing by IL-2–activated NK cells in vitro; if anything, killing was slightly reduced. The killing of the tumor cells in vitro, whether they expressed p53 or not, was nevertheless largely NKG2D dependent, as it was much reduced when the NK cells were derived from Klrk1\(^{−/−}\) mice (Fig. 3 B). Similarly,
blocking NKG2D with antibodies to prevent ligand recognition resulted in a significant decrease in the sensitivity of the tumor cells to killing by NK cells in vitro (Fig. 3 C). Therefore, although p53 restoration did not induce or increase the expression of NKG2D ligands, the elimination of p53-restored tumor cells was dependent on NKG2D recognition by NK cells, as tested by in vitro killing (Fig. 3) or tumor rejection in vivo (Fig. 1).

A subsequent investigation of tumor cell phenotypes in vivo provided another line of evidence that NKG2D plays a role in elimination of the senescent tumor cells. As already noted, p53 restoration did not cause a change of RAE-1ε expression in tumors implanted in Rag2−/−Klk1−/− mice (Fig. 2 B). In Rag2−/− mice, in contrast, p53 restoration for 8 d resulted in a reproducible reduction in the expression of RAE-1ε on tumor cells (Fig. 4, A and B). The reduction was not observed in Rag2−/−Klk1−/− control recipients, or in Rag2−/− mice that were depleted of NK cells (Fig. 4, A and B). Furthermore, the reduction in RAE-1ε expression did not occur in Rag2−/− mice when the tumors were not induced to express p53 (Fig. 4 B). These data suggested that p53 expression facilitates NK cell-dependent tumor cell killing in vivo, resulting in the preferential elimination of tumor cells expressing high levels of the NK activating ligand RAE-1ε.

**p53 restoration is associated with increased chemokine expression and NK cell recruitment**

Having observed no effect of p53 on expression of NKG2D ligands or sensitivity of tumor cells to NK-mediated lysis in vitro, we considered the possibility that p53 expression might either enhance NK cell accumulation in the tumor or the activity of the NK cells once within the tumor. To address this issue, we analyzed mRNA expression of cytokines and chemokines in p53-on or p53-off tumors (day 7 after cultivating the cells in doxycycline). Significance was tested with two-way ANOVA and Bonferroni’s tests at each effector to target ratio. Values represent means ± SEM for each effector to target ratio. The data presented are representative of three independent experiments. (A) Lysis by IL-2-activated NK cells of p53-off versus p53-on target cells (day 7 after cultivating the cells in doxycycline) in the presence or absence of 50 µg/ml NKG2D Ab (clone MI-6) to block the interaction. The significance of the differences between the MI-6-treated groups compared with the control groups was tested with two-way ANOVA and Bonferroni’s tests at each effector to target ratio. Values represent means ± SEM for each effector to target ratio. The data presented in A and B are representative of two independent experiments. Note that error bars are presented throughout but are in some cases smaller than the symbols. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 3. NKG2D-dependent killing of proliferating and senescent tumor cells. (A) Lysis by IL-2-activated NK cells of p53-off versus p53-on tumor cells (day 7 after cultivating the cells in doxycycline). Significance was tested with two-way ANOVA and Bonferroni’s tests at each effector to target ratio. Values represent means ± SEM for each effector to target ratio. (B) Lysis of p53-off and p53-on target cells by WT versus Klrk1−/− IL-2-activated NK cells. Values represent means ± SEM for each effector to target ratio. The data presented in A and B are representative of three independent experiments. (C) Lysis by IL-2-activated NK cells of p53-off versus p53-on tumor cells (day 7 after cultivating the cells in doxycycline) in the presence or absence of 50 µg/ml NKG2D Ab (clone MI-6) to block the interaction. The significance of the differences between the MI-6-treated groups compared with the control groups was tested with two-way ANOVA and Bonferroni’s tests at each effector to target ratio. Values represent means ± SEM for each effector to target ratio. The data presented are representative of two independent experiments. Note that error bars are presented throughout but are in some cases smaller than the symbols. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
A comparable analysis of ex vivo tumor samples from mice treated or not with doxycycline showed a similar induction of mRNAs for these chemokines and several others, including CCL3, CXCL1, and CXCL2 (Fig. 6 B). Many of these changes were confirmed at the protein level using protein arrays (Fig. 6 C).

We next investigated which cells (tumor or immune cells) were producing these soluble factors in vivo when p53 was restored. The transformed tumor cells, which express GFP, were used for tumor implantation in mice. Established tumors were harvested (p53-off and –on) and single cell suspensions of the tumors were stained with CD45 antibodies to stain all hematopoietic cells within the tumor. CD45+ cells and GFP+ cells (tumor cells) were sorted by flow cytometry and tested by Q-RT-PCR. mRNAs for the CC-chemokines CCL2, CCL3, CCL4, and CCL5 and the CXC-chemokines CXCL1 and CXCL2 were found to be up-regulated in GFP+ tumor cells (Fig. 6 D) after p53 expression in vivo (Fig. 6 E). Some up-regulation of the same chemokines may also occur in CD45+ hematopoietic cells in the tumors (Fig. 6 E), but the combined data from three experiments failed to reach statistical significance.

Published chromatin immunoprecipitation experiments show a direct association of p53 with the Cd2 gene regulatory sequences (Hacke et al., 2010; Tang et al., 2012), which is consistent with the possibility that p53 directly transactivates Cd2. Furthermore, the Ct values from the Q-RT-PCR experiments suggested that the tumors produced greater amounts of CCL2 than of the other chemokines tested (unpublished data). To confirm the generality of Cd2 induction in senescent cells by p53, we transduced WT or Trp53−/− (p53 KO) MEFs with H-RasV12 and cultured the cells for 8 d (Fig. 7 A). These experiments were based on a previous study (Brady et al., 2011) showing that H-RasV12 induces senescence in MEFs that express the wild-type form of p53.
Similar to the results with liver tumor cells, a fourfold increase in Ccl2 gene expression was observed in WT MEFs transduced with H-RasV12 (Fig. 7 B). In contrast, H-RasV12 transduction induced much less Ccl2 mRNA in p53 KO MEFs, suggesting that p53 plays a critical role in inducing Ccl2 gene expression (Fig. 7 B). The Ccl2 induction was also confirmed at the protein level by ELISA performed on the cell culture supernatants. As shown in Fig. 7 C, the amount of CCL2 protein secreted by H-RasV12–transduced WT MEFs was much higher than the amount secreted by H-RasV12–transduced p53 KO MEF.

We next investigated the potential role of the secreted CCL2 in the migration of NK cells in vitro, using a transwell system. As shown in Fig. 7 D, H-RasV12–transduced p53–expressing senescent MEFs strongly induced the migration of IL-2–activated NK cells. The migration was blocked when neutralizing CCL2–specific polyclonal antibodies were included in the cultures, demonstrating the role of CCL2 in the observed migration. Importantly, much less migration occurred with H-RasV12–transduced, p53 KO MEF. These data indicate the importance of p53 in the migration process by virtue of its activity in inducing CCL2 production.

Neutralization of CCL2 prevents NK cell recruitment in vivo and diminishes tumor cell rejection

NK cells can be rapidly recruited to sites of infection or inflammation due to expression of various chemokine receptors, including CCR2, CCR5, CXCR3, and CX3CR1 (Grégoire et al., 2007; Walzer and Vivier, 2011). CCR2 is the receptor for CCL2, whereas CCR5 is the receptor for CCL3, 4, and 5. To test the role of these chemokines in leukocyte recruitment and subsequent tumor clearance, mice with implanted tumors were treated with doxycycline and simultaneously treated with control IgG, a neutralizing antibody for CCL2, or a cocktail of neutralizing antibodies for CCL3, CCL4, and CCL5 (Fig. 8). These antibodies have been shown to be effective in neutralizing the corresponding chemokines in published experiments (Martinez de la Torre et al., 2007; Pace et al., 2012).

8 d after administering the antibodies, the percentages of various CD45+ cell types in the tumors were determined. Neutralizing CCL2 caused a striking reduction in the recruitment of NK cells into the tumors, whereas neutralizing CCL3, CCL4, and CCL5 had no such effect (Fig. 8, A and B). The reduction was apparent when NK cell content was expressed...
as the percentage of CD45+ cells (Fig. 8A) or as the percentage of all cells in the tumor (Fig. 8B). In contrast, neutralizing CCL2 (or CCL3, CCL4, and CCL5) had no significant effect on the recruitment of various myeloid cells, including granulocytes (F4/80−Ly6G+CD11b+Ly6C−CD11c−), inflammatory monocytes (F4/80+Ly6G−CD11b+Ly6C+CD11c−), or monocytes/macrophages (F4/80+Ly6G−CD11b+Ly6C+CD11c+; Fig. 8A and B), and consequently did not significantly...
alter the percentage of total CD45+ cells in the tumors (not depicted). These data indicate that CCL2 plays an important and nonredundant role in recruitment of NK cells into senescent tumors, supporting the conclusion that induced expression of CCL2 as a result of p53 expression enhances CCL2-dependent NK cell recruitment to the tumors.

Importantly, the neutralization of CCL2 not only inhibited NK cell recruitment into the tumors, it also delayed the rejection of senescent tumor cells in vivo, as shown by parallel monitoring of tumor sizes after initiating p53 restoration and antibody treatments (Fig. 9). The delay in tumor elimination was significant though not as robust as the delay that occurred in mice treated with NK1.1 antibodies to deplete NK cells. Collectively, the data support the conclusion that induction of CCL2 expression as a result of p53 restoration substantially enhanced NK cell recruitment to tumors, as well as NK cell–dependent elimination of tumor cells mediated by NKG2D interactions.

In addition to the effects of p53 on chemokine expression, the expression data we obtained suggested a role of p53 in the induction of cytokines that are known to activate NK cells (Fig. 10). Thus, p53 restoration resulted in enhanced expression, at both the mRNA and protein levels, of IL-15, IL12p40, IL-18, IL-1, and IL-6 in ex vivo samples of tumors in which p53 expression was restored (Fig. 10, B and C). Analysis of separated tumor cells and infiltrating CD45+ cells indicated that IL-12, IL-15, and IL-1, at least, were induced significantly in tumor cells, whereas IL-6 was probably produced by CD45+ cells (Fig. 10 D). Of the cytokines that were induced in senescent tumor cells, at least IL-15 and IL-12 + IL-18 (which act synergistically) are known to be potent activators of NK cells. Hence, cytokines induced in senescent tumors may enhance NK cell activation within the tumors, aiding in tumor elimination.
DISCUSSION

In this study, we have investigated the mechanistic basis of extrinsic immune-mediated tumor suppression associated with cellular senescence. Previous studies from our laboratory demonstrated that transplanted cells expressing NKG2D ligands are rejected by NK cells and, in some cases, T cells (Diefenbach et al., 2000, 2001, 2003), and that knock-out mice lacking NKG2D display an enhanced incidence and/or more rapid onset of cancers in certain mouse models of spontaneous cancer (Guerra et al., 2008). Published studies showed that NK cells, via granule exocytosis, play a major role in eliminating tumors that become senescent (Xue et al., 2007; Sagiv et al., 2013). Here, we demonstrated that NKG2D-mediated recognition is largely if not exclusively responsible for the NK cell–dependent component of senescent liver tumors. The activation of tumor elimination mechanisms by senescent tumors may benefit the host in three respects: by reducing the likelihood that variant tumor cells lacking p53 can arise from the senescent tumor mass, by facilitating the elimination of passenger nonsenescent tumor cells in the tumor mass, and by preventing the senescent tumor cells from exerting protumorigenic effects on neighboring tissues (Krtolica et al., 2001; Parrinello et al., 2005; Coppé et al., 2006, 2008; Liu and Hornsby, 2007; Läberger et al., 2012).

Considering NKG2D’s role, it might have been expected that NKG2D ligands would be increased in senescent tumors compared with growing tumors. We observed instead that p53 restoration did not increase RAE–1 expression above the already high level we observed in growing tumor cells. These findings are consistent with previous studies suggesting that NKG2D ligands are induced by a variety of other stimuli, including E2F transcription factors, PI3 kinase, Ras oncogene signaling, and an activated DNA damage response (Gasser et al., 2005; Tokuyama et al., 2011; Jung et al., 2012). We had previously concluded that p53 does not play a major role in the expression of NKG2D ligands, at least in the mouse cells tested. In contrast, two studies reported that pharmacological reactivation of p53 in specific cell lines can stimulate the expression of ULPB2, a ligand for human NKG2D (Li et al., 2011; Testor et al., 2011). Hence, p53 may in some cases enhance NKG2D ligand expression, but it appears that others, possibly more important, roles in facilitating NKG2D-dependent elimination of tumors, as documented in this paper.

The main phenotype observed after p53 restoration in vivo was a nearly threefold overall increase in the percentage of immune cells within the tumor, including an increase of $>20$-fold in the percentage of NK cells in the tumors. The accumulation of immune cells was independent of NKG2D. Consistent with a role for senescence in recruiting immune cells, another study showed that mouse livers harboring premalignant senescent hepatocytes showed an inflammatory reaction with large clusters of immune cells surrounding senescent hepatocytes (Kang et al., 2011). Several cytokines (including CCL2) were directly secreted by the senescent hepatocytes. This study correlated the presence of these soluble factors with immune surveillance and infiltration by immune cells (Kang et al., 2011), but direct evidence for their participation was not provided.

Senescent cells and cells expressing p53 communicate with their environment in part by secreting various cytokines, chemokines, and growth factors. We propose that these chemokines play a critical role in the immune surveillance of senescent tumor cells by NK cells and possibly other immune cells. We observed a strong induction of various important chemokines, as well as NK cell–activating interleukins including IL–12 and IL–15 after p53 induction in vitro and in vivo. Our data suggest that some of these chemokines and cytokines were produced directly by senescent tumor cells, whereas tumor-infiltrating immune cells also produced some of these cytokines/chemokines and in one case were probably the exclusive source of a cytokine (IL–6). We were, however, unable to detect IFN–γ production in NK cells extracted from the tumors by flow cytometry, or by using protein arrays with extracts of whole tumors. We also did not observe an increase in IFN–γ mRNA, as examined by Q–RT–PCR. analysis of either whole tumor extracts or extracts of tumor-infiltrating CD45$^+$ cells. These data suggest that the steady-state amounts of IFN–γ produced by NK cells in the tumor are low, lessening the likelihood that increases in IFN–γ production play a major role in tumor rejection in this model.

Although p53-expressing senescent tumors secreted several chemokines with the potential to recruit NK cells, the in vitro migration experiments and in vivo antibody neutralization studies presented herein suggest that CCL2 plays a predominant role in this process. CCL2 was first identified as a monocyte attractant, but evidence has accumulated that it can recruit other cells including memory T cells and dendritic cells. It has been implicated in NK cell recruitment before but has not stood out as the major chemokine for recruiting these cells (Grégoire et al., 2007). The C/EBPα gene, which may be directly transactivated by p53 (Hacke et al., 2010; Tang et al., 2012), was induced in liver tumor cells as a result of p53 restoration and in p53–expressing but not Ip53–KO MEFs transduced with H-RasV12 (Fig. 6, D and E; and Fig. 7, B and C, respectively). In fact, calculations of the relative amounts of CCL2 mRNA in liver tumor cells versus hematopoietic cells of ex vivo tumor cells, which take into account the greater abundance of transformed versus hematopoietic cells in the tumors, suggest that tumor cells account for $>85$% of the CCL2 transcripts (unpublished data). The p53 dependence of CCL2 production may distinguish CCL2 from some of the other chemokines produced in senescent cells, which are dependent on the senescent state but are produced independently of p53 (Coppé et al., 2008; Rodier et al., 2009). The latter chemokines are among the mediators associated with the so-called senescence–associated secretory phenotype (SASP), some of which have protumorigenic activities.

An interesting feature of our findings is that p53 expression did not enhance NKG2D ligand expression or the sensitivity of the tumor cells to NK lysis, but instead functioned by boosting the recruitment of NK cells to the tumor, and potentially the activation state of the NK cells that reached the...
tumors. These findings may be one explanation for why expression of NKG2D ligands in some cancers is insufficient to trigger NKG2D-dependent elimination of tumors (Jinushi et al., 2003; Guerra et al., 2008; McGilvray et al., 2009; Hilpert et al., 2012). In the case of the liver tumor cells studied here, for example, a high level of RAE-1 on the cells was insufficient to cause elimination of the tumor cells, p53 restoration both stalled the growth of the cells and caused the increased recruitment of NK cells that aided in tumor elimination. Hence, stress-induced signals that induce NKG2D ligands and senescence-specific signals that recruit and activate NK cells can cooperate in affecting the elimination of senescent tumors and possibly other senescent tissues in vivo.

Our findings that p53 acts in part by enhancing the recruitment and activation of NK cells has the additional implication that p53 may also, in some cases, enhance rejection of tumors by NK2G2D-independent recognition mechanisms. Depending on the ligands expressed by the tumor cells studied, different NK-activating receptors may be engaged and trigger cytotoxicity (Vivier et al., 2011). Thus, it is plausible that enhanced recruitment and activation of NK cells resulting from p53 expression is important for rejection of tumor cells that express those ligands, as well as for tumors that express NKG2D ligands.

Our results suggest the importance of p53 expression and senescence in immune surveillance at the early stages of tumorigenesis before p53 is inactivated or lost due to mutation. Conversely, an implication of our findings is that loss of p53 function and/or the bypass of senescence, which occurs in the majority of advanced tumors, can impair immune surveillance of tumors by decreasing the efficiency of NK cell recruitment within the tumor. Methods that restore these p53-mediated functions might therefore be fruitful in enhancing NK cell–mediated tumor elimination (Nardella et al., 2011). Given that the mediators act to recruit NK cells, it might be sufficient to restore p53 activity in only a fraction of the tumor cells in a given tumor to achieve a beneficial effect.

Additional analysis of the senescence–associated events that underlie immune surveillance of premalignant senescent cells is clearly warranted. Further studies are needed to elucidate the different senescence–dependent signals provided by tumor cells that will converge to create a cancer–associated pattern needed to trigger immune surveillance. Understanding and characterizing the mechanisms of senescence surveillance such as the secretory phenotype and the regulation of immune cell effector functions is clearly a new research direction in the senescence field. This line of investigation may uncover new strategies for therapeutic augmentation of immune responses against a broad variety of cancers in the future.

### MATERIALS AND METHODS

**Mice and in vivo procedures.** Rag2−/− and Kdhk1−/− (NKG2D KO) mice were both on the C57BL6 background. The NKG2D KO mice strain is available at The Jackson Laboratory Repository with the JAX Stock No. 022733. Rag2−/− and Kdhk1−/− mice were intercrossed to generate Rag2−/− Kdhk1−/− mice. The Kdhk1 knockout genotypes were determined by genomic PCR (Guerra et al., 2008). To generate subcutaneous tumors, 2 × 10^6 liver tumor cells were subcutaneously injected into the rear flanks of the mice. Tumor volume (in cubic millimeters) was determined by caliper measurement and calculated as length × width^2 × π/6. All animal procedures were performed according to the National Institutes of Health guidelines under protocols approved by the University of California Animal Care and Use Committee.

For NK cell depletion in vivo, mice were injected i.p. with 200 µg NK1.1 antibodies (clone PK136) for two consecutive days before doxycycline treatment and every 4 d thereafter once doxycycline treatment was initiated. For blocking chemokines in vivo, mice were injected i.p. with 100 µg CCL2 polyclonal antibodies or a cocktail (100 µg for each Ab) of CCL3 (clone 39624), CCL4 (clone 46097), and CCL5 (clone 53405) antibodies (all from R&D Systems). Injections were performed 1 d before doxycycline treatment and on day 4 after initiation of doxycycline treatment.

**Cells, antibodies, and reagents.** The liver tumor cells (named TRE Shanghai) used in this study were previously reported (Xue et al., 2007). Cell cultures were performed at 37°C in humidified atmosphere containing 5% CO₂. Liver tumor cells and MEFs were cultivated in DMEM with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.2 mg/ml glutamine, 10 µg/ml gentamycin sulfate, and 20 mM Hepes (referred to below as complete medium). For in vitro experiments, cells were cultured in 100 ng/ml doxycycline (Sigma-Aldrich) in complete DMEM medium and the medium was refreshed every 2 d. In vivo, the drinking water was supplemented with 0.2 mg/ml doxycycline in 0.5% sucrose solution, kept in light-protected bottles, and refreshed every 4 d. IL-2–activated NK cells were prepared by incubating wild-type and Kdhk1−/− C57BL6 splenocytes for 5 d RPMI complete medium with 1,000 U/ml recombinant human IL-2 (National Cancer Institute).

The NKG2D (MI-6) and NK1.1 (PK136) antibodies were prepared and purified in house. The following Abs were purchased from R&D Systems: anti–RAE-1c (clone 205/001), anti–panRAE-1 (clone 186/107), anti-H-60 (polyclonal Ab), and APC-conjugated anti-MULTI-1 (clone 237/104). From eBioscience, we purchased biotinylated anti-Nkp46 (clone 29A1.4), streptavidin (SA)-APC and SA-PECy7, APC-conjugated anti-CD45.1 (clone A20), PerCPCy5.5-conjugated anti-Ly4C (clone HK1.4), PE-conjugated anti-F4/80 (clone BMD8), and PEy6C7-conjugated anti-CD11c (clone N418). From BioLegend, we purchased APC-conjugated anti-NKp46 (clone 29A1.4), BV605-conjugated anti-CD11b (clone MI-70), AF700-conjugated anti-Ly4C (clone 1A8), and APC-conjugated anti-human IgG Fc (clone HP6017). Propidium iodide and CountBright absolute counting beads were purchased from Invitrogen.

**Flow cytometry.** Cells cultivated in vitro were preincubated for 20 min on ice with PBS containing 2-4G2 antibodies to block Fc receptors. Between incubations (and before analysis), cells were washed with PBS containing 0.05% BSA and 0.002% sodium azide. For the first staining step, cells were incubated for 30 min on ice with antibodies conjugated with biotin or fluorochromes. Cells were incubated with fluorochrome-conjugated streptavidin for 30 min on ice when necessary. For staining ex vivo cells, fresh tumor tissues were excised from mice, minced, and dissociated using the gentleMACS Dissociator (Miltenyi Biotec). Dissociated tumor samples were further digested in DMEM containing 200 µg/ml Collagenase IV (Roche) and 20 µg/ml DNase I (Sigma-Aldrich) at 37°C for 45 min. After filtering through a 70-µm nylon mesh, single cell suspensions were stained as described above. Flow cytometry was performed with an LSR II or LSRFortessa flow cytometer (BD). FACSDiva software was used for acquisition of data and FlowJo software was used for analysis (BD).

**NK cell cytotoxicity assay.** NK cell cytotoxicity was determined with a standard 4-h 51Cr-release assay. In brief, 10^4 51Cr-labeled TRE Shanghai cells were dispensed in 100 µl of culture medium in quadruplicates in the wells of V-bottomed 96-well plates. IL-2–activated NK cells were added in 100 µl of culture medium to each well to achieve effector-to-target cell ratios of 9:1, 27:1, or 81:1. For NKG2D blocking in vitro, IL-2–activated NK cells were incubated with 100 µg NKG2D Ab (clone MI-6) for 30 min at room temperature.
temperature before adding to target cells. The microcultures were incubated for 4 h at 37°C in a humidified 5% CO₂ atmosphere, after which the plates were centrifuged and 40 µl of the culture supernatants were collected and mixed with 180 µl OPTIPHASE SUPERMIX (PerkinElmer). The radiation in each sample was counted with an automated β counter (Microbeta TriLux; PerkinElmer). The spontaneous release was in all cases <20% of the maximum release accomplished with detergent. The percentage of specific ³¹Cr-release was calculated according to the following formula: % specific lysis = 100 × (experimental – spontaneous release)/(detergent release – spontaneous release).

RNA isolation, reverse transcription, and quantitative PCR. Total RNA was isolated using the RNeasy Mini kit (Qiagen), and treated with DNaseI (DNA-free kit; Invitrogen) for 25 min at 37°C. For comparisons among samples, an equal amount of RNA was reverse transcribed to cDNA using the iScript reverse transcription system (Bio-Rad Laboratories) according to the manufacturer's instructions. Quantitative real-time PCR was performed using SSO-Fast EvaGreen Supermix (Bio-Rad Laboratories) on a CFX96 thermocycler (Bio-Rad Laboratories), according to the manufacturer's instructions. mRNA amounts were normalized to amplifications of the reference 18S rRNA and are plotted as relative expression values. The primers used for Q-RT-PCR analyses are listed in Table S1.

**REFERENCES**


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Hilpert, L. 2007. Senescent human fibroblasts increase the
Krizhanovsky, V., M. Yon, R.A. Dickins, S. Hearn, J. Simon, C. Miething,


example of natural killer cells. Science. 331:44–49. http://dx.doi.org/10.1126/science.1198687


