

# CD160 is essential for NK-mediated IFN- $\gamma$ production

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NK-derived cytokines play important roles for natural killer (NK) function, but how the cytokines are regulated is poorly understood. CD160 is expressed on activated NK or T cells in humans but its function is unknown. We generated CD160-deficient mice to probe its function. Although CD160<sup>-/-</sup> mice showed no abnormalities in lymphocyte development, the control of NK-sensitive tumors was severely compromised in CD160<sup>-/-</sup> mice. Surprisingly, the cytotoxicity of NK cells was not impaired, but interferon- $\gamma$  (IFN- $\gamma$ ) secretion by NK cells was markedly reduced in CD160<sup>-/-</sup> mice. Functionally targeting CD160 signaling with a soluble CD160-Ig also impaired tumor control and IFN- $\gamma$  production, suggesting an active role of CD160 signaling. Using reciprocal bone marrow transfer and cell culture, we have identified the intrinsic role of CD160 on NK cells, as well as its receptor on non-NK cells, for regulating cytokine production. To demonstrate sufficiency of the CD160<sup>+</sup> NK cell subset in controlling NK-dependent tumor growth, intratumoral transfer of the CD160<sup>+</sup> NK fraction led to tumor regression in CD160<sup>-/-</sup> tumor-bearing mice, indicating demonstrable therapeutic potential for controlling early tumors. Therefore, CD160 is not only an important biomarker but also functionally controls cytokine production by NK cells.

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Abbreviations used: BTLA, B and T lymphocyte attenuator; HVEM, herpesvirus entry mediator.

NK cells play multiple roles during the innate immune response, reacting to a myriad of challenges, including pathogen-infected cells, transplanted allogeneic cells, and tumor cells (Moretta et al., 2002; Lanier, 2005). These responses are tightly regulated through multiple activating and inhibitory receptors. Several structurally distinct receptors have been implicated in activating effector functions, including NKp46, NKG2D, 2B4 (CD244), and CS1 (CRACC; Sentman et al., 2006; Marcenaro et al., 2011). Unlike these ubiquitously expressed NK receptors, the CD160 receptor is selectively expressed on the fraction of NK cells with the highest cytotoxic functions (Maïza et al., 1993).

CD160 is an immunoglobulin-like, glycosylphosphatidylinositol-anchored protein with homology to killer-cell immunoglobulin-like receptors (Agrawal et al., 1999). In addition to its association with effector function, CD160 was demonstrated to bind broadly to MHC class I molecules with low affinity, first in humans

(Barakonyi et al., 2004) and later in mice (Maeda et al., 2005). A recent study, however, demonstrated that human CD160 binds to herpesvirus entry mediator (HVEM), a TNF family member, with much higher affinity than to MHC class I, and leads to suppressed T cell responses in vitro (Cai et al., 2008). Whether this high-affinity interaction exists in vivo and what role it plays remains unclear.

HVEM has been shown to regulate both the innate and adaptive responses through its multiple binding partners, both as a ligand and as a receptor. Via B and T lymphocyte attenuator (BTLA) on T cells, the delivery of HVEM is largely inhibitory, controlling T cell effector responses (Sedy et al., 2005; Deppong et al., 2006) and the innate response (Sun et al., 2009). In contrast, signaling through HVEM activates T cells by LIGHT/TNFSF14 (Cheung et al.,

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2005; Cai and Freeman, 2009). However, the nature of the HVEM–class I MHC–CD160 interactions has not been well defined *in vivo*. To directly address these questions, we generated CD160<sup>-/-</sup> mice and soluble CD160 (CD160-Ig) fusion protein and investigated the necessity and sufficiency of CD160 on the effector function of NK cells *in vivo* and *in vitro*. We reveal here that CD160 is a functional regulator of cytokine production by NK cells and is important for early control of tumor growth.

## RESULTS

### Generation of CD160-deficient mice

To define the role for CD160 *in vivo*, we generated a mouse strain on the C57BL/6 background with a targeted mutation of the CD160 gene (Fig. 1 A). In this strain, exon 2, which contains the initiation codon and which is required for all known splice variants (Giustiniani et al., 2009), was replaced with a Neo cassette. Removal of exon 2 also rendered the downstream exons out of frame, ensuring the absence of any CD160 protein sequence. We confirmed by electrophoresis that no exon 2–containing CD160 transcripts existed in our KO strain, and that primers amplifying regions spanning exon 2 were the correct size for transcripts lacking this exon (Fig. 1 A). The molecular weights for the WT and KO Southern bands were 11,183 and 8,408 bp, respectively. To verify the loss of CD160 protein expression in our CD160<sup>-/-</sup> mouse, splenocytes from WT and CD160<sup>-/-</sup> mice were labeled with fluorescence-coupled CD160 mAb or isotype control. Consistent with previous works (Maeda et al., 2005; Rabot et al., 2006, 2007), resting NK cells from WT mice expressed limited amounts of surface CD160. However, when NK cells were stimulated with increasing concentrations of IL-2, the surface expression of CD160 was significantly elevated in an IL-2 dose-dependent manner within 18 h of stimulation, a response consistent with a previous study (Fig. 1 B; Le Bouteiller et al., 2002). In comparison, NK cells from the CD160-deficient mice had no detectable CD160 expression at all doses of IL-2 tested (Fig. 1 B). This not only confirmed the lack of CD160 protein in genetically deficient mice, but also suggested that CD160 may play a regulatory role in NK activity.

Using this CD160<sup>-/-</sup> strain, we determined whether CD160 deficiency alters the frequency of T cells, NK cells, or NKT cells, each of which have been previously reported to express CD160 (Agrawal et al., 1999; Le Bouteiller et al., 2002; Barakonyi et al., 2004; Maeda et al., 2005; Rabot et al., 2006; Cai et al., 2008). Total numbers of splenocytes, splenic T cells, NK cells, and NKT cells, as well as thymic T cell subsets were comparable between WT and CD160<sup>-/-</sup> mice (Fig. 1, C and D). Therefore, it appears that CD160 does not play a significant role in the generation of T cells or NK cells.

### CD160 is required for early control of NK-dependent tumors

To determine whether NK function is altered in the absence of CD160, we used two distinct NK-dependent tumor models: the B16 mouse melanoma model and the RMA-S

lymphoma model. The B16 melanoma model requires NK cells for early control of tumor growth (Kakuta et al., 2002). When inoculated subcutaneously with B16 tumor cells, CD160<sup>-/-</sup> mice developed tumors of significantly larger sizes than WT mice (Fig. 2 A). To determine whether CD160 is required specifically by NK cells for control of tumor growth, we depleted NK cells before tumor inoculation by injection of NK1.1 mAb. After NK cell depletion, WT mice supported significantly faster growing tumors, whereas CD160<sup>-/-</sup> mice depleted of NK cells had similar tumor growth kinetics to untreated mice (Fig. 2 A). These data suggest that CD160 is required for NK-mediated B16 tumor challenge.

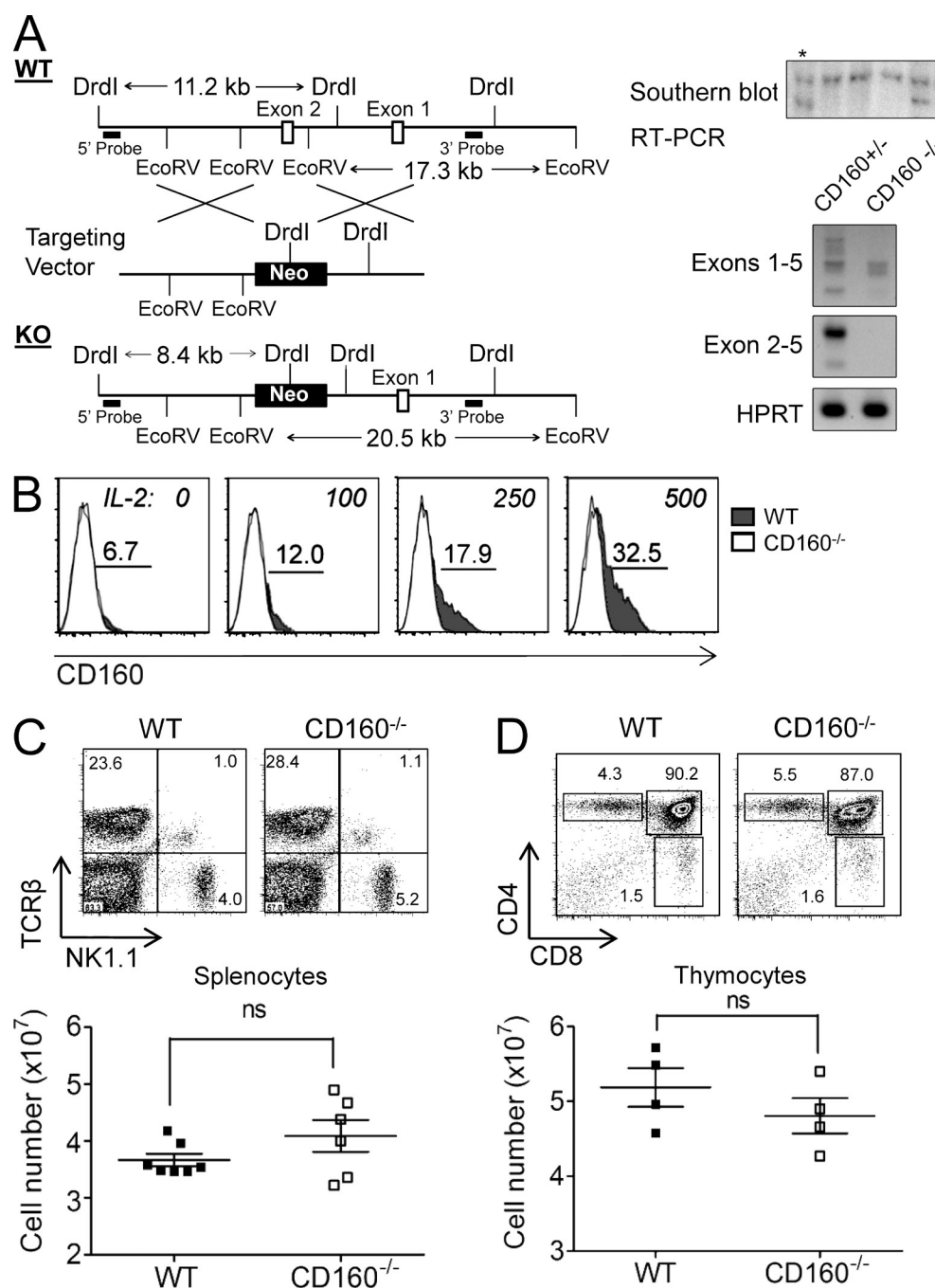
As CD160 has been shown to be up-regulated on not only NK cells but also T cells after activation, we crossed CD160<sup>-/-</sup> mice with RAG1<sup>-/-</sup> mice to focus on the potential role of CD160 on NK cells in the absence of adaptive immune system. Similar to naive CD160<sup>-/-</sup> mice, no apparent defects in total numbers of splenocytes or splenic NK cells were observed in naive CD160/RAG<sup>-/-</sup> mice (Fig. 2 B). However, B16 tumors grew significantly larger in CD160/RAG<sup>-/-</sup> mice, compared with RAG<sup>-/-</sup> controls (Fig. 2 C), demonstrating that, in the absence of T cells, CD160 is still necessary for a robust anti-tumor immune response to B16 melanoma.

To further investigate the role of CD160 in the NK cell response to tumors, we adapted the RMA-S lymphoma line to our subcutaneous tumor model. Because RMA-S cells have a defect in MHC class I assembly, thus lacking surface MHC class I, they make suitable targets for NK cells both *in vivo* and *in vitro* (Kärre et al., 1986; Ljunggren et al., 1991; Smyth et al., 1998). RMA-S inoculated subcutaneously into CD160/RAG<sup>-/-</sup> mice grew significantly faster and larger than RAG<sup>-/-</sup> controls, demonstrating that CD160-deficient NK cells were profoundly impaired in clearing RMA-S tumor cells *in vivo* (Fig. 2 D). Furthermore, the difference in tumor growth was no longer observed in mice inoculated with the parental RMA lymphomas, which express intact MHC class I and therefore do not activate NK cells (Fig. 2 E). These data demonstrate that CD160 is specifically required for an optimal antitumor immune response primarily mediated by NK cells.

Because we had observed CD160's induction on NK cells after *in vitro* stimulation (Fig. 1 B), we next determined whether CD160 were induced in response to *in vivo* tumor burdens. As such, we inoculated RMA-S tumors in WT or CD160<sup>-/-</sup> mice, and harvested tumor tissues and splenocytes after the tumor-burden reached a designated size of 500 mm<sup>3</sup>. Consistent with our previous observation of CD160 induction after *in vitro* stimulation (Fig. 1 B), we also observed an increase in CD160 expression on NK cells after *in vivo* stimulation in tumor-bearing mice compared with naive mice (Fig. 2 F). This suggests that CD160 may exert its role systemically, as represented by the spleen in response to a subcutaneous tumor.

### CD160 is not required for NK-mediated cytotoxicity but required for cytokine production

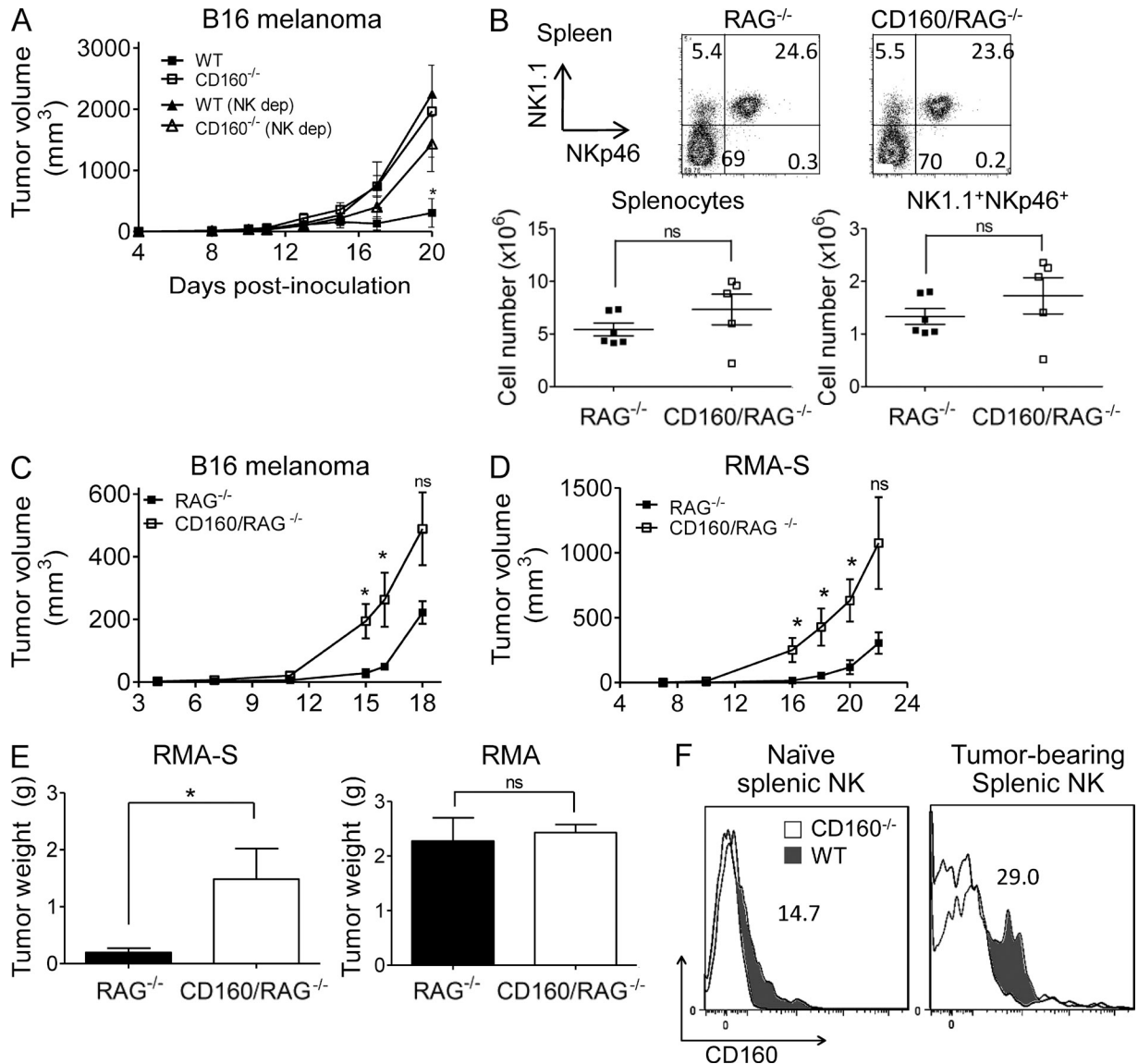
Despite having normal numbers of NK cells, CD160<sup>-/-</sup> mice were susceptible to early outgrowing tumors. Therefore, we



**Figure 1. Generation and characterization of CD160<sup>-/-</sup> mice.** (A) Schematic of CD160 targeting vector. (top) Relevant portion of WT mouse chromosome 3, targeting vector, and genomic sequence after homologous recombination. Exon 1, exon 2 (replaced by Neo sequence in KO), and DrdI and EcoRV restriction sites used for typing by Southern blot are shown. (bottom) 5' Southern blot verifying mutant gene. \*, correctly targeted clones successfully used to make CD160<sup>-/-</sup> founder lines. (right) Electrophoresis by Southern and Northern blots of CD160 exons from CD160<sup>+/-</sup> and CD160<sup>-/-</sup> splenocytes. The molecular weights for the WT and KO Southern bands were 11,183 and 8,408 bp, respectively. (B) Splenocytes from WT and CD160<sup>-/-</sup> mice were cultured in complete medium supplemented with increasing concentrations of rIL-2 (0, 100, 250, and 500 U/ml). After 18 h, cells were harvested and labeled with NK1.1, TCRβ, and CD160 mAbs. FACS histograms show NK1.1<sup>+</sup>TCRβ<sup>+</sup> cells. Numbers in FACS plots indicate percentages of gated populations of WT cells. Splenocytes (C) or thymocytes (D) were harvested and labeled with TCRβ and NK1.1 or CD4 and CD8 mAbs, respectively. Cell populations were gated as shown in FACS plots and cell numbers were calculated from total cell counts. Data are representative of three independent experiments.

evaluated the functional role of CD160 on NK cells. First, we evaluated whether NK cytotoxicity was impaired in CD160<sup>-/-</sup> NK cells. We used the chromium release assay of

YAC-1 target cells, which are highly sensitive to direct NK killing upon cell-to-cell contact with NK cells. We radiolabeled the target cells and co-cultured them with freshly isolated



**Figure 2. CD160 is required for controlling NK-sensitive tumors.** (A) WT and CD160<sup>-/-</sup> mice were inoculated subcutaneously with 10<sup>5</sup> B16 melanoma cells. NK-depleted mice were administered NK1.1 mAb 1 d before tumor inoculation, and weekly thereafter. \*, significant differences ( $P < 0.05$ ) at days 17 and 20 between WT and CD160<sup>-/-</sup> groups, and WT and WT (NK dep) groups. Differences between CD160<sup>-/-</sup> and CD160<sup>-/-</sup> (NK dep), and WT (NK dep) and CD160<sup>-/-</sup> (NK dep) were not significant. Each group contained five mice. Data are representative of three independent experiments. (B) Splenocytes from RAG<sup>-/-</sup> and CD160/RAG<sup>-/-</sup> mice were labeled with NK1.1 and Nkp46 mAbs. Cell populations were gated as shown in FACS plots and cell numbers were calculated from total cell counts. Data are representative of three independent experiments. (C) RAG<sup>-/-</sup> and CD160/RAG<sup>-/-</sup> mice were inoculated subcutaneously with 5 × 10<sup>5</sup> B16 melanoma cells. \*, significant differences ( $P < 0.05$ ) between groups at days 15 and 16. Each group contained 5 mice. Data are representative of three independent experiments. (D) For the RMA-S tumor model, RAG<sup>-/-</sup> and CD160/RAG<sup>-/-</sup> mice were inoculated subcutaneously with 5 × 10<sup>5</sup> RMA-S. \*, significant differences ( $P < 0.05$ ) between RMA-S groups at days 16, 18, and 20 by tumor volume. (E) 5 × 10<sup>5</sup> RMA-S or RMA lymphoma cells were inoculated subcutaneously as before, and weights of tumors resected at day 22 after inoculation were measured. \*, significant difference ( $P < 0.05$ ) between RMA-S groups at day 22 by tumor weight. (F) The expression of CD160 on NK cells from tumor-bearing mice was determined by flow cytometry. Shown are cells gated from the 7-AAD<sup>-</sup>, CD3<sup>-</sup>, NK1.1<sup>+</sup> population. Mice were inoculated with 2 × 10<sup>6</sup> RMA-S tumor cells, and tumors were allowed to grow until 500 mm<sup>3</sup> before termination. Lymphocytes from the spleen were harvested into single-cell suspension, and labeled with NK1.1, TCRβ, and CD160 mAbs as previously described. Data are representative of three independent experiments.

splenocytes from naïve RAG<sup>-/-</sup> and CD160/RAG<sup>-/-</sup> mice. We found no defect in cytotoxicity of CD160-deficient mice, indicating that CD160 is not required for NK-mediated cytotoxicity (Fig. 3 A). The intact killing activity despite CD160

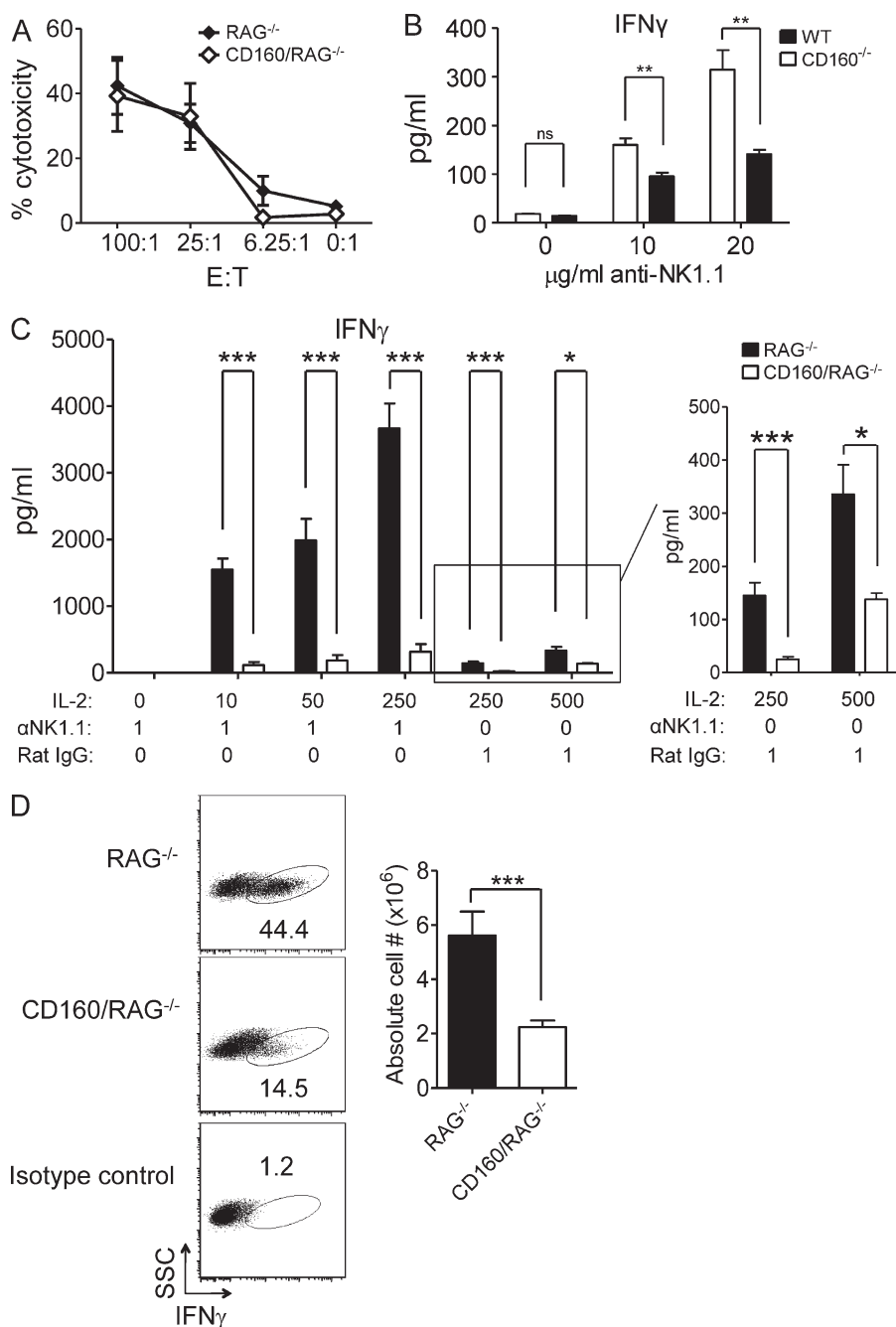
deficiency suggests that the mice in our tumor models may not be controlling tumor growth by means of cytotoxicity.

The activity of an NK cell is thought to depend on cumulative signals delivered by activating and inhibitory signals,

with greater engagement of activating receptors favoring NK activation (Vivier et al., 2004). Cross-linking of the surface NK1.1 receptor with its specific mAbs results in activation of NK cells and increased IFN- $\gamma$  production (Kim et al., 2010). Therefore, we next determined whether the cytokine response was impaired in NK cells from CD160-deficient mice. To this end, we stimulated splenocytes from WT or CD160 $^{-/-}$  mice plate-bound anti-NK1.1 mAb, and measured the level of IFN- $\gamma$ . While WT splenocytes demonstrated strong IFN- $\gamma$  production, CD160 $^{-/-}$  splenocytes demonstrated poor IFN- $\gamma$  production upon NK1.1 cross-linking (Fig. 3 B). This impaired IFN- $\gamma$  response of NK cells in the absence of CD160

was also observed in our CD160/RAG $^{-/-}$  mice and could not be rescued by the addition of high doses of IL-2, as increasing the concentration of IL-2 even up to 250 U/ml in culture failed to restore the level of IFN- $\gamma$  to that of controls (Fig. 3 C).

To confirm that the cytokine defect was specifically within the NK population, we performed intracellular cytokine staining on NK cells after in vivo priming and in vitro stimulation, which revealed reduced IFN- $\gamma$  within the NK1.1 $^{+}$  population of lymphocytes (Fig. 3 D). These results suggested that NK cells do require CD160 for optimal IFN- $\gamma$  production. Additionally, equal numbers of NK cells persisted throughout these activation assays, with no differences in viability



**Figure 3. NK cells require CD160 for IFN- $\gamma$  production, but not killing.** (A) The percent cytotoxicity from  $^{51}\text{Cr}$  release assay are shown for total splenocytes freshly isolated from naive RAG $^{-/-}$  or CD160/RAG $^{-/-}$  mice and cultured with radiolabeled YAC-1 target cells for 4 h at the indicated effector-to-target ratios. Data are representative of three independent experiments. (B) Splenocytes from naive WT and CD160 $^{-/-}$  mice were cultured at  $2 \times 10^5$  cells per well in 96-well flat bottom plates with complete medium supplemented with rhIL-2 (100 U/ml) and plate-bound anti-NK1.1 at indicated concentrations. After 48 h, culture supernatants were harvested and IFN- $\gamma$  concentration was determined. (C) Splenocytes from naive RAG $^{-/-}$  and CD160/RAG $^{-/-}$  mice were cultured as in B, with indicated concentrations of IL-2 and either plate-bound anti-NK1.1 or isotype control antibodies. After 48 h, culture supernatants were harvested and IFN- $\gamma$  concentration was determined. (D) For intracellular cytokine staining, RAG $^{-/-}$  and CD160/RAG $^{-/-}$  mice were primed with intraperitoneal injections of poly(I:C) (100  $\mu$ g) 18 h before splenocytes were harvested and cultured for 6 h in complete media containing Brefeldin-A. During this 6-h culture, cells received rhIL-2 (100 U/ml) and plate-bound anti-NK1.1 mAb (1  $\mu$ g/ml). (left) Representative FACS plots; (right) quantification of the absolute numbers of IFN- $\gamma$ -producing NK cells. Intracellular cytokine staining was performed using IFN- $\gamma$ , NK1.1, and Nkp46 mAbs. \*,  $P < 0.05$ . Each group contained five mice. Data are representative of three independent experiments. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Each group contained splenocytes pooled from 5 naive mice.



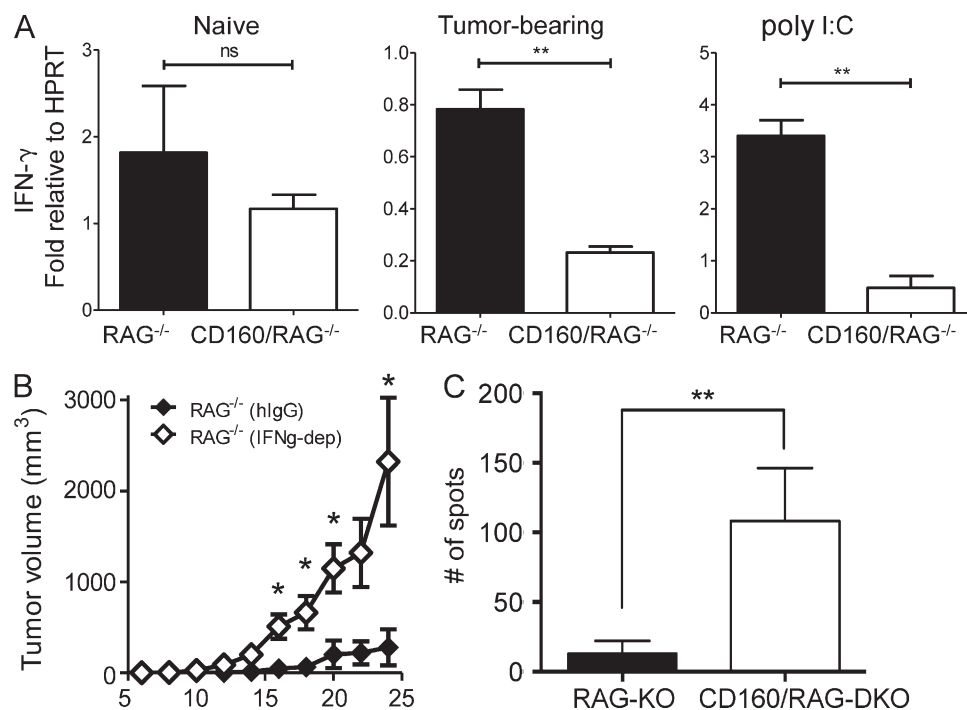
between  $RAG^{-/-}$  and  $CD160/RAG^{-/-}$  mice (unpublished data), suggesting that CD160 may be regulating the effector function rather than cell viability or exhaustion of NK cells. Finally, we used real-time PCR to determine IFN- $\gamma$  expression, which does not require ex vivo stimulation. In splenocytes from naive mice, we found no impact of CD160 deficiency on IFN- $\gamma$  expression (Fig. 4 A). However, in tumor-bearing or poly(I:C)-treated mice, CD160-deficient mice had significantly reduced expression of IFN- $\gamma$  (Fig. 4 A). This suggested that CD160 is required for cytokine production in activated NK cells.

Finally, we asked whether the reduced IFN- $\gamma$  in mice lacking CD160 could directly result in outgrowing tumors in vivo. We neutralized IFN- $\gamma$  by administering a depleting anti-IFN- $\gamma$  mAb alongside B16 tumor inoculation and weekly i.p. injections thereafter. As a result, mice receiving neutralizing mAb had significantly faster growing tumors compared with isotype control (Fig. 4 B). Additionally, by reducing the numbers of tumor cells inoculated, we observed that IFN- $\gamma$ -neutralized hosts had significantly higher rates of tumor engraftment (unpublished data). This suggests that IFN- $\gamma$  is important for early control of tumors, likely by NK cells, and opens the possibility that NK cells can control tumors

with cytokines rather than cytotoxicity. In addition, to assess whether CD160 was required for controlling metastasis, we used a melanoma model in which B16 tumor cells were delivered retro-orbitally into  $RAG^{-/-}$  or  $CD160/RAG^{-/-}$  mice, and lung tissues were observed for metastatic nodules. We observed significantly increased metastatic nodules in the  $CD160/RAG^{-/-}$  recipients (Fig. 4 C). Together with the inability to rescue activation by IL-2 or NK1.1 engagement, our data suggests that CD160 regulates essential NK activation pathways, leading to the production of IFN- $\gamma$ .

#### CD160 defines a novel cytokine-producing arm of NK cells

To determine whether CD160 could be a marker for the cytokine-producing NK cell subset, we used CD160 to divide the NK population from  $RAG^{-/-}$  control mice into  $CD160^{+}$  versus  $CD160^{-}$  subsets. When stimulated with increasing dosage of IL-2 together with plate-bound anti-NK1.1, the  $CD160^{+}$  subset produced significantly greater IFN- $\gamma$  than the  $CD160^{-}$  subset (Fig. 5 A). This demarcation of IFN- $\gamma$ -producing capacity could also be elicited by in vivo stimulation of  $RAG^{-/-}$  mice with poly(I:C) followed by in vitro stimulation with PMA/ionomycin (Fig. 5 B), indicating that CD160 functionally defines an NK subset endowed with IFN- $\gamma$ -producing capacity.



**Figure 4. IFN- $\gamma$  is essential for CD160-mediated tumor control in vivo.** (A) Spleens were harvested from  $RAG^{-/-}$  and  $CD160/RAG^{-/-}$  mice from three conditions: naive, tumor-bearing (day 22 post-tumor inoculation), and poly(I:C) (24 h after 100  $\mu$ g i.p.). RNA was isolated from splenocytes by TRIzol extraction, reverse transcribed into cDNA, and quantitated by real-time PCR for IFN- $\gamma$  and HPRT. All data in this figure are representative of three independent experiments. (B) IFN- $\gamma$  is required for early tumor control. Mice were inoculated with  $5 \times 10^5$  B16 melanoma cells subcutaneously. These mice were also intraperitoneally administered the anti-IFN- $\gamma$  mAb (XMG1.2). Antibody treatments were continued on a weekly basis during tumor growth. Tumor volumes were measured every 2 d. (C) To assess control of tumor metastasis, tumor cells were intravenously delivered into recipient mice. Naive  $RAG^{-/-}$  and  $CD160/RAG^{-/-}$  were retro-orbitally injected with  $3 \times 10^5$  B16 melanoma cells. After 14 d, mice were sacrificed and lungs were harvested. Black spots in the lungs were counted and graphed. \*\*,  $P < 0.005$ . All data presented here are representatives of three independent experiments.

To determine whether the IFN- $\gamma$ -producing NK subset delineated by CD160 was important for tumor control and to evaluate its therapeutic potential, we transferred purified populations of CD160-expressing NK cells versus CD160 non-expressing NK cells intratumorally into RMA-S tumors of CD160 $^{-/-}$  mice. To achieve clear separation of CD160 $^{+}$  versus CD160 $^{-}$  NK populations, donor mice were first primed with poly(I:C), as previously described. The CD160 $^{-/-}$  mice regained control of tumor growth soon after receiving CD160 $^{+}$  NK cells compared with CD160 $^{-/-}$  mice receiving CD160 $^{-}$  NK cells (Fig. 5 C). This indicates that the NK subset defined by CD160 is not only functional *in vitro*, but also helps control tumors *in vivo* and demonstrates the therapeutic potential of CD160 in the treatment of cancer.

Because CD160 expression can be induced after NK activation, we determined whether the NK subset defined by CD160 could maintain stable expression of CD160, and also whether the CD160-negative NK cells could induce new CD160 expression. After NK activation, the CD160 $^{+}$  fraction of NK cells maintained CD160 expression (Fig. 5 D). However, the CD160 $^{-}$  fraction acquired CD160 expression, and began expressing significant levels comparable to the initially positive fractions by 6 d after culture. Interestingly, before day 4 of stimulation, there was no detectable expression of CD160 in the CD160 $^{-}$  fraction. This suggests a degree of stability to CD160 expression, particularly for our *in vitro* assays evaluating CD160 $^{+}$  and CD160 $^{-}$  NK fractions. However, after prolonged stimulation, NK cells can acquire new expression of CD160.

### CD160 functionally regulates the NK response

Although absolute numbers of NK cells were not affected by CD160 deficiency, the possibility still exists that the defect seen in CD160-deficient mice *in vivo* could have been caused by issues of NK cell development or homeostasis. As such, to avoid the possible effect of CD160 during NK development, we generated a murine CD160-Ig fusion protein to functionally block CD160 interactions *in vivo*, serving as a soluble decoy receptor to CD160's natural ligands. Our CD160-Ig contained the mature extracellular CD160 sequence with mammalian posttranslational modifications, linked with the Fc portion of human IgG1.

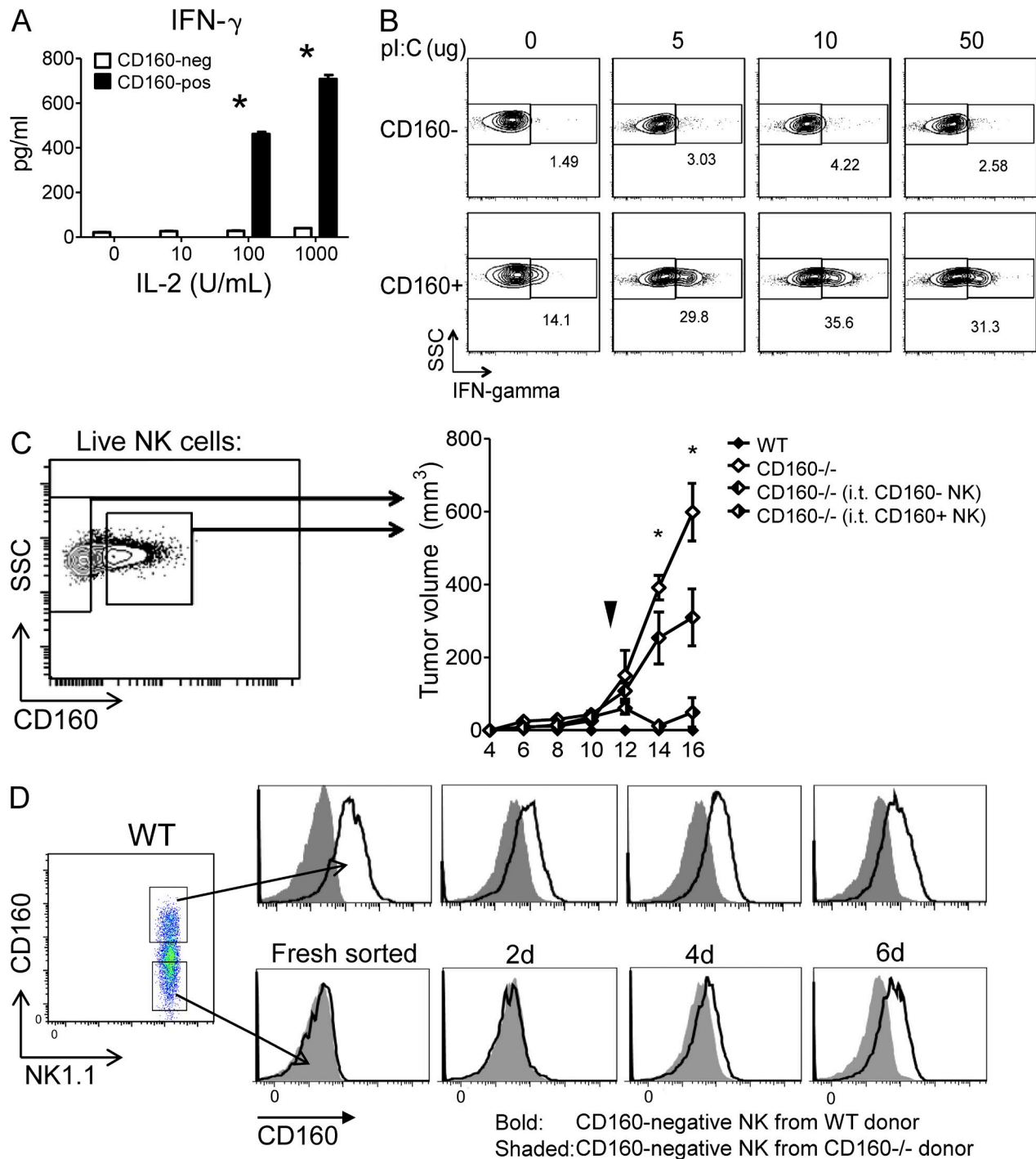
To functionally target the CD160 pathway, we administered a low dose of CD160-Ig intratumorally in RAG $^{-/-}$  tumor-bearing mice as soon as tumors reached >75 mm $^3$  in size. The CD160-Ig-treated tumors grew larger than isotype control-treated tumors (Fig. 6 A, left). Splenocytes harvested from CD160-Ig-treated B16 melanoma-bearing mice were also significantly impaired for IFN- $\gamma$  production after NK1.1 activation *ex vivo* (Fig. 6 A, right). To reduce the potential bias from differential tumor sizes between CD160-Ig and isotype-treated groups, we also determined the IFN- $\gamma$  response relatively early after tumor inoculation, before the tumor sizes diverged (Fig. 6 B, left). Similarly between both early and late stages of tumor burden, we saw marked reduction in levels of IFN- $\gamma$  after *ex vivo* NK activation (Fig. 6 B, right). Similar defects were observed when CD160-Ig was used in the

RMA-S lymphoma model of RAG $^{-/-}$  mice. Analogous to the B16 response, the RMA-S tumors also grew faster and larger after CD160-Ig intratumoral administration (Fig. 6 C, left). Correspondingly, the splenic IFN- $\gamma$  response after NK activation was also reduced in both early and late stages of tumor burdens (Fig. 6 C, right). Finally, to determine whether CD160-Ig treatment could impact the tumor response in CD160/RAG $^{-/-}$  mice, we intratumorally administered the fusion protein as previously described alongside both control RAG $^{-/-}$  and CD160/RAG $^{-/-}$  mice (unpublished data). Although CD160-Ig treatment compromises tumor control in RAG $^{-/-}$  mice, the treatment did not alter the tumor growth of CD160/RAG $^{-/-}$  mice, suggesting that CD160-Ig functions as a blocking rather than activating molecule *in vivo*. These data demonstrate that targeting the CD160 pathway in the otherwise unremarkable RAG $^{-/-}$  host could impair tumor control and IFN- $\gamma$  production.

However, these experiments testing CD160-Ig used tumor-bearing mice and smaller tumor burden in treated group might influence the result. To determine whether CD160-Ig could also impair cytokine production independently of tumor burden, we administered CD160-Ig *i.p.* together with poly(I:C). Poly(I:C) was used to prime NK cells because such priming is required for *ex vivo* NK function and CD160-Ig had no effect over isotype control in naive mice (unpublished data). When we measured cytokine production 18 h after treatment, we observed significantly reduced IFN- $\gamma$  production in CD160-Ig treated mice after *ex vivo* stimulation by plate-bound anti-NK1.1 and IL-2, as well as in freshly isolated splenocytes without additional stimulation (Fig. 6 D). These data further confirm that CD160 functionally regulates cytokine production in activated NK cells.

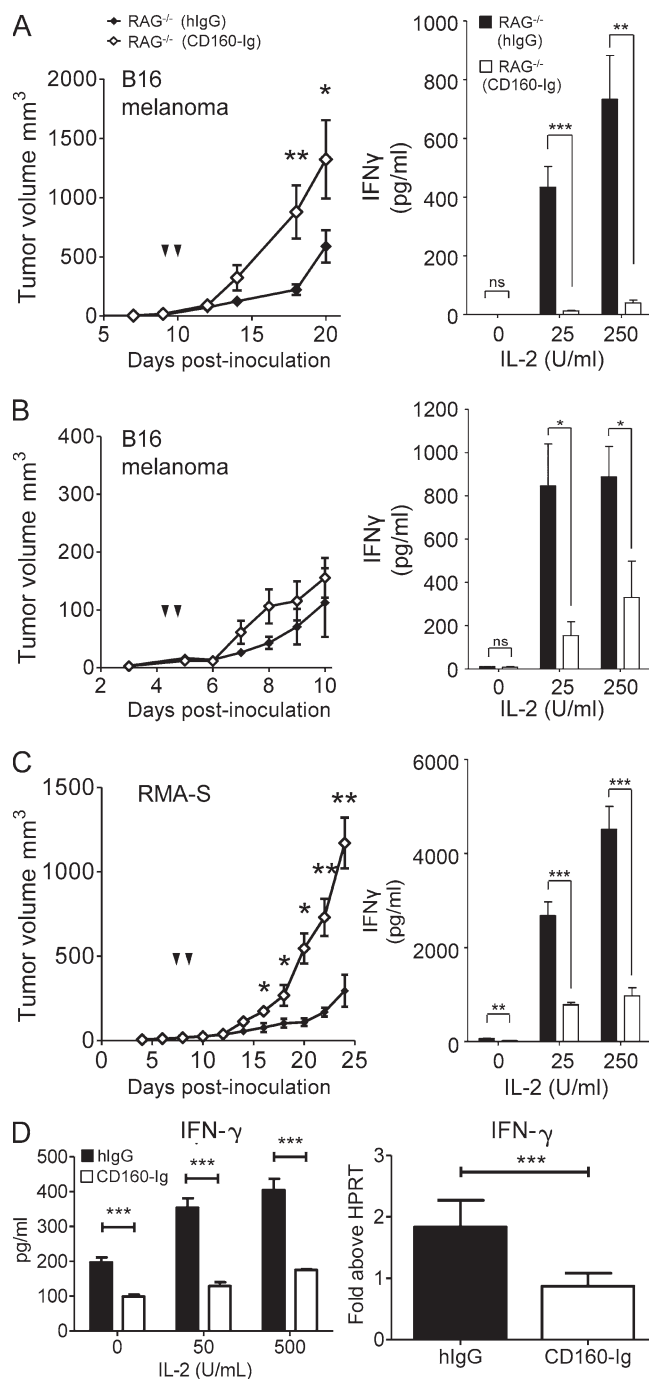
### CD160 is intrinsically required by NK cells for efficient cytokine production

CD160 is required by NK cells for efficient production of IFN- $\gamma$ . To determine whether this requirement occurs through a cell-intrinsic or -extrinsic mechanism, we generated mixed BM chimeric mice. We reconstituted lethally irradiated WT (CD45.1) hosts with equal ratios of WT (CD45.1) and CD160 $^{-/-}$  (CD45.2) BM cells. After reconstitution, we evaluated IFN- $\gamma$  production as before, using poly(I:C) to prime the chimeric mice before *ex vivo* stimulation with PMA/ionomycin. We did not observe preferentially reduced NK numbers or percentage in the CD45.2 fraction over the CD45.1, further confirming that CD160 deficiency does not significantly impair NK development. However, intracellular cytokine staining revealed that the CD45.2 $^{+}$  CD160 $^{-/-}$  NK cells produced significantly lower IFN- $\gamma$  than CD45.1 $^{+}$  WT NK cells even in the same recipients. Dividing the IFN- $\gamma$  producers into low or high fractions, we further observed the reduction in the CD160 $^{-/-}$  NK cells consistently across both fractions (Fig. 7 A). In addition to a 1:1 chimera, we generated a chimera which was reconstituted with primarily (90%) CD160 $^{-/-}$  BM. In these chimera, we also observed that CD160 $^{-/-}$  NK cells produced even less IFN- $\gamma$  per cell despite outnumbering the WT



**Figure 5. CD160 defines the cytokine-producing subset of NK cells.** (A) To obtain pure populations of CD160<sup>+</sup> versus CD160<sup>-</sup> NK cells, splenocytes freshly isolated from naive RAG<sup>-/-</sup> mice were sorted by flow cytometry for NK1.1 and CD160 expression into two fractions: NK cells expressing or lacking CD160. The two NK fractions were stimulated with increasing dosages of IL-2 together with plate-bound anti-NK1.1 (1  $\mu$ g/ml), and supernatants were measured for cytokines after 48 h in culture. (B) We determined the cytokine production capacity of these subsets by priming mice with poly(I:C) for 18 h before harvesting splenocytes and restimulating the NK with PMA/ionomycin. Splenocytes were surface stained for NK1.1 and CD160, and fixed/permeabilized to stain IFN- $\gamma$  intracellularly. (C) Donor RAG<sup>-/-</sup> mice were primed with poly(I:C) (100  $\mu$ g, i.p.) for 18 h before harvesting of splenocytes, which were then stained for NK1.1 and CD160 for sorting. Sorted fractions of CD160-expressing or CD160-non expressing NK cells were intratumorally injected into RMA-S tumors of CD160<sup>-/-</sup> mice at  $5 \times 10^5$  cells per 100 mm<sup>3</sup>-sized tumors. Tumor sizes were measured every 2 d. (D) To determine the stability of CD160 expression on CD160<sup>+</sup> versus CD160<sup>-</sup> NK cells, WT and CD160<sup>-/-</sup> naive mice were sacrificed, and freshly isolated splenocytes sorted by FACS for CD3<sup>+</sup>NK1.1<sup>+</sup>CD160<sup>-</sup> and CD3<sup>+</sup>NK1.1<sup>+</sup>CD160<sup>+</sup> populations. CD160<sup>+</sup> and CD160<sup>-</sup> populations from both WT and CD160<sup>-/-</sup> donors were cultured with IL-2 (500 U/ml) for 2, 4, and 6 d. At each time point, cells were harvested, washed, and stained for CD160 expression. Representative FACS plots are shown to indicate CD160 expression levels over the 6 d of culture. Data are representative of three independent experiments.





**Figure 6. CD160 functionally regulates the NK response to tumor.**

Groups of mice were inoculated with B16 and RMA-S tumors and received intratumoral injections of CD160-Ig or hlgG isotype control when tumor sizes reached ~75–100 mm<sup>3</sup> in volume, followed by a second injection 3 d later. The fusion proteins were prepared in matrigel suspension at 50 µg/ml for each injection per tumor. (A) B16 tumor sizes were monitored. Figures on the left indicate tumor volumes, while figures on the right indicate the IFN-γ production by splenocytes harvested from tumor-bearing mice corresponding to the end of the tumor curve (day 20 post-inoculation) and cultured with anti-NK1.1 with increasing concentrations of IL-2 for 48 h. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (B) Here, the B16 tumor model was halted when the tumors were under 200 mm<sup>3</sup> in volume,

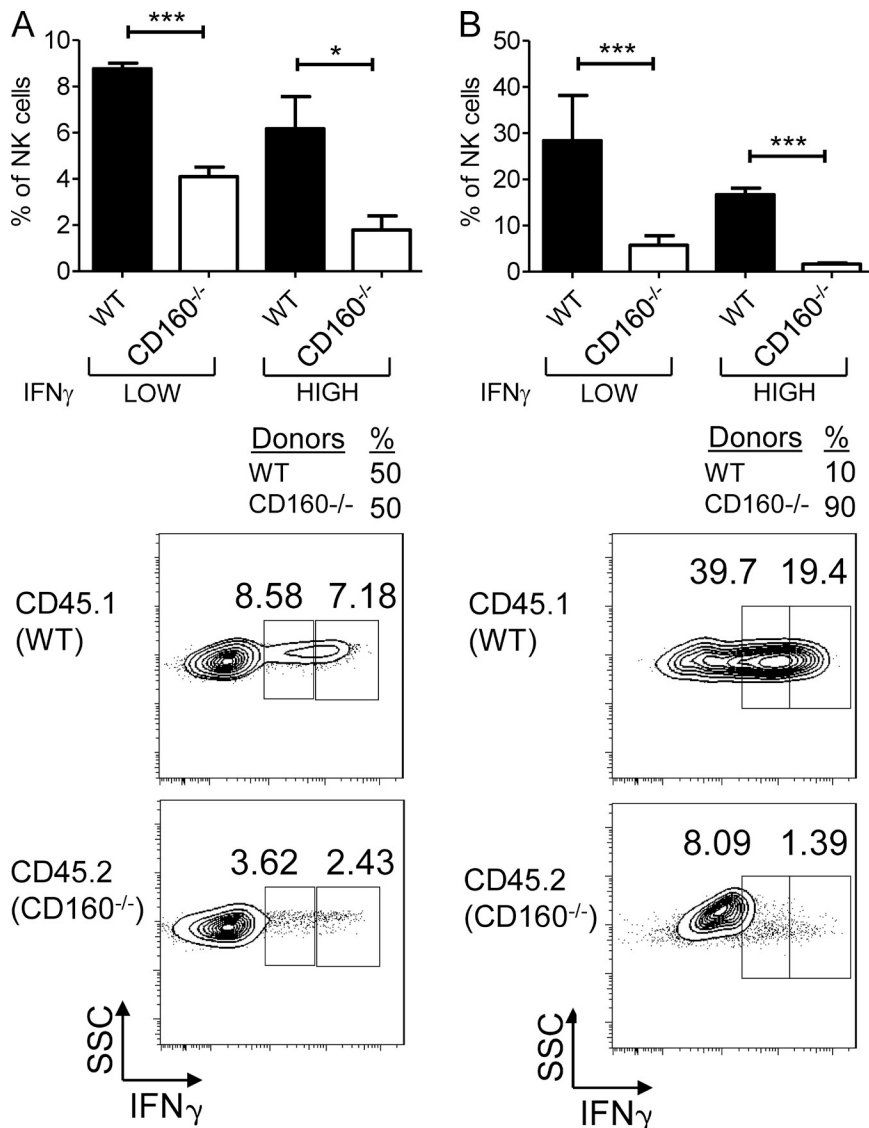
NK cells by almost 10-fold (Fig. 7 B). Together, these results demonstrate that CD160 is required intrinsically by NK cells for efficient IFN-γ production.

#### NK-CD160 coordinates with non-NK-HVEM to regulate IFN-γ

The binding partner to CD160 has remained elusive due in part to the promiscuous arrangement of competing binding partners. CD160 was first shown to bind to classical and non-classical MHC class I molecules (Maeda et al., 2005), and was later shown to bind to HVEM with higher affinity (Cai et al., 2008). We have no evidence that CD160-Ig binds physiologically to class I molecules in vivo. To test whether CD160 can bind to HVEM, we used our CD160-Ig reagent, and demonstrated binding of CD160-Ig to 293-HEK cells transfected with HVEM (Fig. 8 A). To further confirm that CD160 interacts with HVEM, we developed an ELISA-based approach using HVEM-Ig and CD160-Ig fusion proteins. We demonstrated CD160's interaction with HVEM, using plate-bound mouse HVEM-Ig to capture soluble CD160-Ig. CD160-Ig was bound in a dose-dependent manner over isotype control (Fig. 8 B). Together, these data suggest that CD160 directly engages HVEM.

Because we demonstrated that CD160 is required on the NK cell, we next sought to determine whether HVEM is required on a partner cell. Because our methods for eliciting IFN-γ required poly(I:C) priming in vivo, we tested whether the DC or macrophage which express TLR-3 could provide the HVEM to engage CD160 on the NK cell. Consistent with RNA data from the public ImmGen database, we also observed that HVEM is most highly expressed on DCs and macrophages (unpublished data). As such, we developed an in vitro co-culture system to combine purified NK cells with DC/macrophages cultured from BM cells grown in GM-CSF. To determine whether HVEM on DC/macrophages were required to induce CD160-dependent IFN-γ, we co-cultured RAG<sup>-/-</sup> or CD160/RAG<sup>-/-</sup> purified NK cells together with WT or HVEM<sup>-/-</sup> BM-derived DC/macrophage. We observed that HVEM was required on the DC/macrophage as NK cells co-cultured with HVEM<sup>-/-</sup> DC/macrophage produced significantly less IFN-γ than WT controls (Fig. 8, C and D). These data, together with our in vivo poly(I:C) primed data suggest that CD160 regulates NK function through coordinating with HVEM on DC/macrophage accessory cells.

which was at day 10 post-inoculation, followed by harvesting of splenocytes for NK activation to measure the IFN-γ response. (C) The RMA-S lymphoma model was used instead of B16, and the experiment was performed analogously to Figure 6A. (D) Naive RAG<sup>-/-</sup> mice were administered CD160-Ig or hlgG isotype control (50 µg) together with poly(I:C) (50 µg) intraperitoneally. After 18 h, spleens were harvested and stimulated ex vivo by plate-bound anti-NK1.1 and IL-2 as described before. Splenocytes were also analyzed by RT-PCR as described before. All data shown are each representative of 3 independent experiments. \*\*\*,  $P < 0.01$ .



**Figure 7. CD160 is required intrinsically by NK cells for IFN- $\gamma$  production.** (A) To determine whether CD160 regulates NK cells intrinsically or extrinsically, we generated mixed BM chimeric mice. We used the C57BL/6 strain of mice bearing the congenic marker CD45.1 to demarcate WT hematopoietic cells from the CD45.2 expressing CD160 $^{-/-}$  cells. BM ( $2 \times 10^6$  cells) from WT (CD45.1) and CD160 $^{-/-}$  (CD45.2) donors were transferred at an equal ratio (1:1) into lethally irradiated (1,000 Rad) WT (CD45.1) recipients. Validation of chimerism was performed by staining peripheral blood lymphocytes. After 6 wk of reconstitution, mice were sacrificed and splenocytes were stimulated *in vitro* by PMA/ionomycin for intracellular cytokine staining. (A) The percentage of IFN- $\gamma$ -producing cells are quantitated in the histograms, with representative FACS plots below, showing the IFN- $\gamma$  staining gated on either CD45.1 $^{+}$  or CD45.2 $^{+}$  CD3 $^{+}$ NK1.1 $^{+}$  cells. The fraction of IFN- $\gamma$  $^{+}$  NK cells were further divided into low versus high, as quantitated in the histograms above. (B) In addition to chimeras with equal ratio of hematopoietic grafts, we generated chimeras in which the dominant hematopoietic source was deficient of CD160 (90% CD160 $^{-/-}$ , 10% WT). As in A, histograms quantitating the percentage of IFN- $\gamma$ -producing NK cells are above the representative FACS plots. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.0005$ . Data are representative of three independent experiments.

## DISCUSSION

CD160 was first identified in a cytotoxicity screen to select lymphocytes with the highest cytotoxic capacity from human peripheral blood (Maïza et al., 1993), and has served as a marker for selecting NK cells and CTLs that can kill target cells more efficiently than their CD160 $^{-}$  brethren (Barakonyi et al., 2004; Maeda et al., 2005; Fons et al., 2006; Tsujimura et al., 2006; Cai et al., 2008). Although this correlation with killing suggests that CD160 might play a stimulatory role, the engagement of CD160 by HVEM was shown to suppress the T cell response, raising the possibility that CD160 could also have inhibitory roles (Cai et al., 2008). Whether CD160 functionally regulates the cytotoxic lymphocytes has remained inconclusive, in part because of the lack of a genetic model. Here, we demonstrate that genetic deficiency of CD160 impaired the function of NK cells to produce cytokines, indicating that CD160 is functionally important for a specific arm of the NK effector function. Alongside the knockout model,

we also showed that soluble CD160 could reduce the cytokine production by NK cells. Furthermore, we demonstrated that this cytokine response is an essential component of the innate response to control tumor growth. This was unexpected because cytotoxicity has long been considered the primary effector mechanism of NK cells (Trinchieri, 1989; Moretta et al., 2001, 2002; Yokoyama and Plougastel, 2003; Yokoyama, 2005). As such, our newly demonstrated functional role of CD160 highlights the cytokine arm of killer lymphocytes.

Our experiments demonstrated that CD160 was required for efficient IFN- $\gamma$  production in activated NK cells, whereas resting NK cells were not impacted by CD160 deficiency. This suggested that CD160 functions as a co-stimulatory molecule for promoting NK cytokine production after a primary stimulus, such as poly(I:C) priming or NK1.1 cross-linking. One potential mechanism could be through receptor synergy. In our NK activation assays, we used plate-bound anti-NK1.1 mAb to agonize the NK1.1 receptor, which signals through the FcR $\gamma$

for its signal transduction possibly involving PKC- $\theta$  and CD45, which were shown to be essential in triggering transcription of IFN- $\gamma$  without affecting the cytotoxicity. Similarly, cross-linking CD160 was shown to promote Akt and PI3K phosphorylation (Rabot et al., 2007; Liu et al., 2010). Because NK1.1 is enriched at lipid rafts (Fassett et al., 2001) and CD160, as a GPI-anchored protein, is likely also enriched at lipid rafts (Rabot et al., 2007), CD160 could be converging with NK1.1 to enhance the downstream FcR $\gamma$ , PKC- $\theta$ –CD45, and Akt–PI3K pathways to promote IFN- $\gamma$  production. (Arase et al., 1997; Vivier et al., 2004; Vahlne et al., 2008). Therefore, in the absence of CD160, the primary NK1.1-mediated stimulus to the NK cell might be less efficient, resulting in less IFN- $\gamma$ .

Interestingly, given the recent reports that PKC- $\theta$  and CD45 tyrosine phosphatase impacts IFN- $\gamma$  secretion but not cytotoxicity in NK cells, CD160 may preferentially signal through PKC- $\theta$  and/or CD45 (Tassi et al., 2008; Hesslein et al., 2006) downstream of NK1.1 activation to induce transcription of IFN- $\gamma$ . This model of lipid raft-mediated receptor synergy is consistent with the current view of NK regulation, i.e., that NK cells depend on cumulative signals from activating and inhibitory receptors (Vivier et al., 2004). Based on this model, when an NK cell engages a target, receptor synergy is important for determining the outcome.

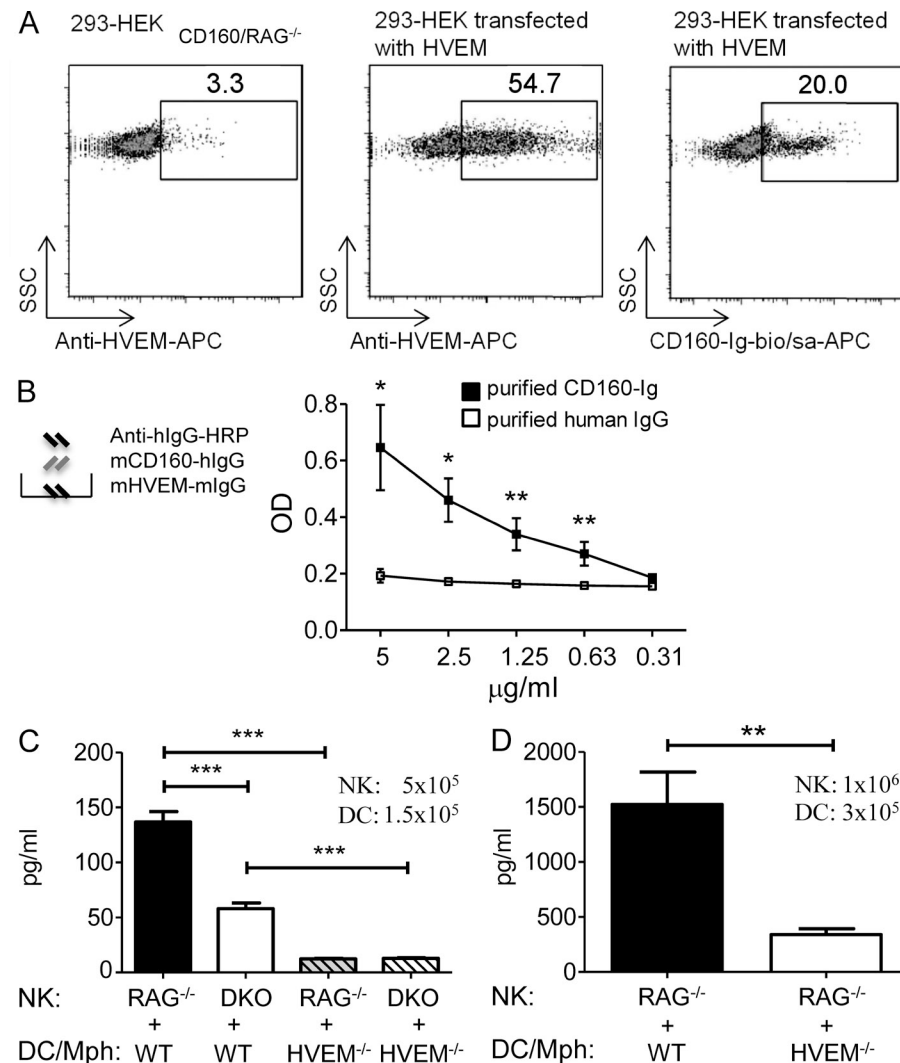
Additionally, the model of receptor synergy is also supported by our demonstration that CD160 is required intrinsically by the NK cell. Although the other binding partner to HVEM, BTLA, has been shown to signal bi-directionally as both a receptor and ligand, our mixed BM chimeras showed that CD160 functions as an NK receptor, but not as a ligand, because WT NK cells that expressed CD160 were not able to rescue CD160<sup>-/-</sup> NK cells within the same host. As such, this supports a revised model in which CD160 is a receptor on NK cells that receives signals from the widely expressed molecules HVEM and/or MHC-I. Additionally, whereas BTLA is expressed on human NK cells, we and others have observed significantly reduced BTLA expression on mouse NK cells (Han et al., 2004; Hurchla et al., 2005; Iwata et al., 2010; D'Addio et al., 2013). This discrepancy between mouse and human BTLA expression helps elucidate the role of CD160 in our mouse model by removing one potential binding partner on our NK cells.

By priming the mice with poly(I:C), which indirectly stimulates NK cells by first activating DCs through TLR-3, we observed reduced IFN- $\gamma$  in CD160<sup>-/-</sup> NK cells. This suggests that CD160 on NK cells could coordinate with DCs, which express both MHC-I and HVEM, the putative binding partners to CD160. In this regard, we showed for the first time that HVEM is required on the DC/macrophage compartment by NK cells for poly(I:C)-induced cytokine production. Although other cell types, including NK cells could also provide MHC-I and/or HVEM, we predict that the DC/macrophage compartment is the main partner to CD160<sup>+</sup> NK cells because they express the highest levels of surface HVEM, as well as innate receptors such as TLR-3 that respond to poly(I:C). However, this does not rule out the possibility of

NK-to-NK homotypic interactions, in which CD160<sup>-</sup> NK cells might provide more accessible HVEM than CD160<sup>+</sup> NK cells because BTLA and CD160 has been recently demonstrated to bind to HVEM in cis (Cheung et al., 2009). It has recently been shown that CD160 competes with BTLA for binding to HVEM (Kojima et al., 2011). In addition, poly(I:C) has broad immunological effects that extend beyond the DC. As such, the possibility of stromal cell involvement remains to be determined. Finally, the tumor itself could provide ligands to CD160. However, we demonstrated CD160's function on both B16 and RMA-S tumors, which rules out tumor-derived MHC-I (because RMA-S lack surface MHC-I) as well as HVEM (because B16 lack surface HVEM).

To determine the mechanism underlying the overgrowing tumors in our CD160-deficient mice, we first assessed the cytotoxic capacity of the NK cell. To our surprise, CD160 was not required for killing because CD160-deficient NK cells could kill as well as CD160-sufficient NK cells. This was unexpected because CD160 expression was previously correlated with cytotoxicity (Agrawal et al., 1999; Le Bouteiller et al., 2002). In addition, earlier studies also showed that the capacity of an NK cell to produce cytokines was mutually exclusive with cytotoxicity (Yokoyama, 2005; Blasius et al., 2007). This suggested that CD160 might regulate a select subset of NK cells that is functionally distinct from other CD160<sup>-</sup> NK cells. As such, when we examined the stability of the NK fraction defined by CD160, we found that CD160<sup>+</sup> NK cells remained positive, whereas CD160<sup>-</sup> NK cells gradually became positive after prolonged activation. Furthermore, the CD160<sup>+</sup> NK fraction was endowed with greater cytokine production capacity and could rescue tumors in CD160<sup>-/-</sup> hosts, compared with their CD160<sup>-</sup> brethren. Together, this data suggests that CD160 does not globally regulate the NK compartment, but instead exclusively regulates a distinct subset specialized for cytokine production.

CD160 is required for optimal IFN- $\gamma$  production by NK cells, an underappreciated but critical mechanism for controlling tumors, which we confirmed through IFN- $\gamma$  depletion in tumor-bearing mice. There are several possibilities as to how the NK cytokine response can control tumors. One possibility is that IFN- $\gamma$  can inhibit the tumor cell cycle, and recent studies have shown that IFN- $\gamma$  synergizes with TNF through TNFR1 expressed on tumor cells to induce cell cycle arrest (Kakuta et al., 2002; Müller-Hermelink et al., 2008; Braumüller et al., 2013). A second possibility is that the IFN- $\gamma$ -producing population of NK cells, which express CD160, could directly interact with tumor stroma, as was recently reported in human tumors in which mAb's against CD160 was able to inhibit angiogenesis (Fons et al., 2006). Finally, a third possibility is that the CD160<sup>+</sup> subset of NK cells produce sufficient cytokines, such as IFN- $\gamma$ , to activate other NK cells, lymphocytes, macrophages, and neutrophils to work together at controlling the tumor. This last avenue is highly probable given our finding that CD160 on NK cells may coordinate with HVEM on non-NK cells such as macrophages, which infiltrate the tumor microenvironment over the natural



**Figure 8. HVEM is the functional partner for CD160-dependent IFN- $\gamma$  by NK cells.** (A) The 293-HEK cell line was transiently transfected with an HVEM-expression plasmid using the transfection reagent polyethylenimine (PEI). Surface expression of HVEM was verified by the anti-HVEM mAb (HMHV-1B18). CD160-Ig was biotinylated and tetramerized with streptavidin conjugated with the APC fluorophore, and the fusion protein tetramers were used to stain HVEM-transfected 293-HEK cells. (B, top) schematic of ELISA. Plate-bound mHVEM-mIg was incubated with mCD160-hlg and detected with anti-human IgG. (bottom) Adsorbed mHVEM-mIg was incubated with titrated, purified mCD160-hlg or control hlgG isotype control. (C) To determine whether HVEM was required on DCs to trigger CD160-dependent IFN- $\gamma$  production on NK cells, we co-cultured purified NK cells together with BM-derived DCs at a ratio of 1:0.3 of NK-to-DCs. Co-cultures were stimulated with poly(I:C) (100  $\mu$ g/ml) for 48 h in round-bottom wells, after which supernatants were harvested for detection of cytokines by cytokine bead array. (D) We increased the cell densities while maintaining the same NK/DC ratio as before. The stimulation and measurement proceeded as previously described. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ . Data are representative of three independent experiments.

course of tumor development. Furthermore, a recent study has demonstrated that CD160 demarcates a unique population of intraepithelial lymphocytes (currently classified as ILC-1) that specializes in producing IFN- $\gamma$  (Fuchs et al., 2013). Thus, the role of CD160 on the host production of IFN- $\gamma$  extends from the spleen to the gut. Whether and how the gut-derived IFN- $\gamma$  coordinates with splenic-derived (and other tissue-derived) IFN- $\gamma$  remains to be explored. As such, our study highlights CD160 and HVEM as a new immunological bridge in innate immunity that coordinates NK cells with non-NK cells.

Our animal model opens new possibilities to dissect this complex regulatory network, and establishes a much needed tool to probe how HVEM and CD160 together coordinate the immune response. Our demonstration of CD160's positive regulation of NK cells is unexpected, considering the previously reported inhibitory role of CD160 on T cells (Cai et al., 2008). Understanding this positive axis of CD160 is pivotal for understanding not only how the NK response is regulated, but also how the innate and adaptive responses

together are balanced by HVEM, not to mention the therapeutic potential of CD160 for fighting cancer.

## MATERIALS AND METHODS

**Animals.** WT and RAG1<sup>-/-</sup> C57BL/6 mice were purchased from Harlan Teklad. HVEM<sup>-/-</sup> C57BL/6 mice were described previously (Wang et al., 2005), and were backcrossed onto C57BL/6 background 10 generations, and then further crossed with RAG1<sup>-/-</sup> mice to generate HVEM/RAG<sup>-/-</sup>.

CD160<sup>-/-</sup> mice were generated on the C57BL/6 background at the University of Chicago (Chicago, IL). A targeting vector containing a neo cassette flanked by homology arms to mouse chromosome 3 sequences surrounding CD160 exon 2 was generated by recombineering (Fig. 1 A). This vector was introduced into C57BL/6-derived embryonic stem (ES) cell line PRX-B6N #1 (Primogenix, Inc.) and ES cell clones were screened by PCR and Southern blot to identify correctly recombined clones. 4-cell embryos from CD-1 mice were injected with 6–8 recombined ES cells to produce a mouse completely derived from the C57BL/6 ES cells. The resulting male mouse was mated with C57BL/6j females to produce CD160<sup>+/-</sup> mice with germline transmission of the knockout allele. These mice were bred to homozygosity and the loss of CD160 transcription was verified by reverse-transcription PCR (rt-PCR) and protein expression by flow cytometry (Fig. 1, A and B). Primers used for rt-PCR were as



follows: exon 1, 5'-GCAAGGAAGAATCTGTCCTGAG-3'; exon 2, 5'-CCACAGACCAGCAAAGTCAAC-3'; and exon 5, 5'-CCTCGCTG-TACCTTCTCTG-3'. CD160/RAG<sup>-/-</sup> mice were generated by crossing CD160<sup>-/-</sup> mice with C57BL/6-RAG1<sup>-/-</sup> mice purchased from The Jackson Laboratory. WT (C57BL/6J) mice were also purchased from The Jackson Laboratory. Mice of both sexes were used between 8–12 wk of age. All experiments have been approved by the University of Chicago Institutional Animal Care and Use Committee.

All mice were maintained under specific pathogen-free conditions. Animal care and use were in accordance with Institutional and National Institutes of Health guidelines, and all studies were approved by the Animal Care and Use Committee of the University of Chicago.

**Cell lines.** NK cells were obtained from spleen cell suspensions via negative enrichment, according to the manufacturer's protocol (Miltenyi Biotec). Enriched NK cells were cultured in complete RPMI with indicated concentrations of rhIL-2 (Chiron).

The B16/F10 murine metastatic melanoma cell line was grown on tissue culture plates, using complete RPMI media supplemented with 10% FBS. RMA (MHC class I H-2b positive) and RMA-S (MHC class I H-2b negative) thymoma cells were grown in suspension in complete RPMI supplemented with 10% FBS.

**Tumor models.** Mice were injected subcutaneously with tumor cells in the lower back. Tumor masses were measured by a millimeter caliper. Tumor volume was calculated by multiplying the width, length, and height of the tumor mass and dividing the product by two.

**NK in vitro assays.** NK cells were harvested from spleens and cultured in 96-well flat bottom tissue culture plates. Anti-NK1.1 (PK136) mAbs at the indicated concentrations were coated onto plates 1 d before NK cell culture (Kim et al., 2010). Titrations of IL-2 were added to complete RPMI medium containing 10% FBS.

**Lymphocyte depletion.** Lymphocytes were depleted by intraperitoneal injection of 200 µg of NK1.1 mAb in 100 µl of PBS. Antibodies were injected 1 d before the start of the tumor inoculation, as well as weekly thereafter for 2 wk, resulting in a total of 3 injections per mouse.

**Cytometric bead array (CBA).** Cytokine measurement was performed with the mouse inflammation CBA kit (BD) according to the manufacturer's guidelines.

**Quantitative real-time PCR.** Total RNA from cells were isolated with TRIzol reagent (Invitrogen) and treated with DNase I, followed by reverse transcription with random primers and AMV reverse transcription (Promega). Real-time RT-PCR was then performed with the SsoFast EvaGreen supermix (Bio-Rad Laboratories) on the StepOne Plus (Applied Biosystems).

The primers used for amplification of IFN-γ cDNA were as follows: forward, 5'-AAGCTACACACTGCATCT-3'; reverse, 5'-GAGCTCATTGAATGCTTGG-3'. The primers for HPRT cDNA were as follows: forward, 5'-TGAAGAGCTACTGTAATGATCAGTCA-3'; reverse, 5'-AGCAAGCTTGCAACCTTAACCA-3'. Amplifications (4 s at 95°C and 30 s at 60°C) were performed for 47 cycles. All samples were normalized to HPRT and reported using the 2<sup>-ΔΔC<sub>T</sub></sup> format.

**Cytotoxicity assay.** To measure direct NK-mediated cytotoxicity, we used the standard chromium 51 (<sup>51</sup>Cr). Freshly isolated splenocytes from RAG<sup>-/-</sup> mice were lysed of RBCs, counted, and cultured with YAC-1 target tumor cells. The YAC-1 cells were labeled with 0.75 MBq/ml (20 µCi/ml) for 1 h at 37°C. Effector and target cells were diluted in RPMI-1640 media containing 10% FBS and HEPES buffer and incubated in round-bottom wells for 4 h at 37°C. Spontaneous <sup>51</sup>Cr release was determined by treating YAC-1 cells with media alone, and maximum release was determined by treating YAC-1 cells with Triton X-100 at 2.0% in media. Supernatants were harvested and

transferred to 96-well plates precoated with scintillant (Lumaplate-96; Perkin-Elmer). The counts-per-minute (CPM) were measured using the Microbeta 1450 plate reader (PerkinElmer). The percentage of specific cytotoxicity was calculated as follows: [(sample cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100. The spontaneous releases were <10%, and all experiments were performed in separate triplicate wells.

**Flow cytometry, antibodies, and fusion protein.** Flow cytometry analysis was performed on FACSCalibur, FACSCanto, and FACSaria II instruments (BD) and analyzed using FlowJo software (Tree Star). Antibodies were purchased from BD, BioLegend, Invitrogen, or eBioscience. Intracellular cytokine staining was performed using permeabilization and fixation buffers supplemented with saponin (eBioscience).

CD160-Ig fusion protein was created by joining the extracellular portion of mouse CD160 with the Fc-portion of human IgG1. The DNA sequence encoding expressed CD160 was determined based on the CD160 mature protein sequence. The plasmid containing this sequence was then cloned into a plasmid containing the human IgG1 cassette. Together, the mouse CD160 and human IgG1 plasmid was subcloned into a plasmid containing an Ig-Kappa leader sequence. Finally, Chinese hamster ovary cells were transfected with the final plasmid for protein production.

For fusion protein tetramerization, we first biotinylated the CD-Ig fusion protein or isotype control using the EZ-Link Sulfo-NHS-LC-Biotinylation kit according to the manufacturer's protocol (Pierce). Then, we multimerized the biotinylated monomers according to a previously published protocol (Altman and Davis, 2003).

For intratumoral administration, fusion proteins were resuspended in Matrigel Basement Membrane Matrix (BD). CD160-Ig (50 µg/ml) and hlgG control (50 µg/ml) were resuspended 1:1 with stock Matrigel, before intratumoral injection.

**Statistics.** Comparisons of data were analyzed by two-tailed Student's *t* test using GraphPad Prism 5.0. Data from such experiments are presented as mean values ± SEM. Differences of *P* < 0.05 were considered significant.

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