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Targeted Disruption of the *2B4* Gene in Mice Reveals an In Vivo Role of 2B4 (CD244) in the Rejection of B16 Melanoma Cells[1]

Swapnil V. Vaidya,[2]* Susan E. Stepp,[2†] Megan E. McNerney,[‡] Jae-Kyung Lee,* Michael Bennett,[§] Kyung-Mi Lee,[‡] Colin L. Stewart,[¶] Vinay Kumar,[‡] and Porunelloor A. Mathew[3]*

Murine 2B4 (CD244) is a cell surface receptor expressed on all NK cells, γδ-T cells, a subset of $CD8^+$ T cells, and all $CD14^+$ monocytes. 2B4 binds to CD48 with high affinity, and cross-linking 2B4 with anti-2B4 Ab in vitro causes activation of NK cells. To study its physiological role, we have generated, by gene targeting, mice deficient in the expression of this cell surface molecule. The expression of lymphoid cell surface markers on PBMC and splenocytes of mice homozygous for the mutation in 2B4 ($2B4^{-/-}$) is identical to that in wild-type mice. However, thymocytes from female $2B4^{-/-}$ mice, but not male $2B4^{-/-}$ mice, have an increase in the immature $CD4^-/CD8^-$ population. To investigate the in vivo role of 2B4, wild-type and $2B4^{-/-}$ mice were injected with $CD48^+$ and $CD48^-$ metastatic B16 melanoma cells. Wild-type mice rejected $CD48^+$ melanoma poorly compared with $CD48^-$ tumor cells, suggesting that ligation of 2B4 by CD48 on melanoma cells is inhibitory. In keeping with this, male $2B4^{-/-}$ mice showed enhanced ability to reject $CD48^+$ melanoma cells. However, female $2B4^{-/-}$ mice poorly rejected both $CD48^+$ and $CD48^-$ melanoma cells, revealing a gender-specific and CD48-independent defect in mice lacking 2B4. In vitro and in vivo experiments reveal a complex role of NK cells in gender specificity. *The Journal of Immunology,* **2005, 174: 800–807.**

2B4 is a member of the CD2 subset of the Ig superfamily expressed on all NK cells, γδ T cells, a subset of $CD8^+$ T cells, and all human $CD14^+$ monocytes (1). It was originally identified as an activating receptor on murine NK cells and $CD8^+$ T cells that mediated non-MHC-restricted cytotoxicity (2, 3). Ligation of surface 2B4 with anti-2B4 mAb augments the cytotoxicity of these cells against various tumor cell lines and induces IFN-γ secretion. Similarly, murine γδ T cells were shown to express 2B4, which played a functional role in the killing of skin tumors (4, 5). In murine NK cells, 2B4 is expressed as two isoforms, 2B4-S and 2B4-L, that result from alternative splicing (6). Studies in which 2B4-S and 2B4-L were transfected into a rat leukemia cells line suggested that 2B4-S functions as an activating receptor, whereas 2B4-L is inhibitory (7). Other NK receptors have been shown to have a functionally opposite receptor, with both receptors binding to the same ligand (8–12). CD48 has been identified as the ligand for 2B4 (13, 14). Because CD48 is a common marker expressed by cells of the hemopoietic system, this suggests that 2B4 may play a role in immune regulation. Recent studies have elucidated the function of 2B4 on T cells. 2B4-CD48 interaction between neighboring CD8 T cells induces proliferation by acting as a costimulatory interaction (15). 2B4-CD48 interaction also augments the MHC-restricted killing by CD8 T cells (16) and the natural cytotoxicity of NK cells.[4] Thus, it appears that in mice 2B4 can regulate NK cells by two distinct mechanisms: by 2B4-CD48 interaction among NK cells and by interaction with CD48-expressing tumor cells. How these two are regulated in vivo is unknown. A recent study has shown that NK cells can also augment the proliferation of neighboring T cells through 2B4 on NK cells interacting with CD48 on T cells (17).

Cloning of the human homologue of mouse 2B4 resulted in several studies of the function of this molecule in humans (18–20). Activation of 2B4 on human NK cells by anti-2B4 Ab or CD48 stimulates cytotoxicity, IFN-γ secretion, and invasiveness (20–22). 2B4 has also been implicated in immune regulation and disease. It plays a regulatory role in human NK cell precursors that are differentiated in vitro (23). These precursors acquire the activating receptors and cytolytic potential even before they express MHC class I-specific inhibitory receptors. This suggests that NK precursors may be potentially reactive to the surrounding cells. However, 2B4 acts as an inhibitory receptor at this stage, contributing to the self-tolerance that is exhibited by these precursor cells. The anti-2B4 mAb, C1.7, stains with high avidity $CD4^+$ CTL that are involved in clinical heart rejection (24). Peritt et al. (25) showed that the percentage of 2B4-expressing $CD8^+$ T cells in the periphery of HIV-infected patients is increased compared with that

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[4] K.-M. Lee, J. P. Forman, M. E. McNerney, S. Stepp, S. Kuppireddi, D. Guzior, H. Wei, Y. E. Latchman, M. H. Sayegh, H. Yagita, et al. Requirement of homotypic NK cell interaction through 2B4(CD244)/CD48 in the generation of NK effector funcitons. *Submitted for publication.*

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Table I. *Primers used for PCR cloning of 2B4 gene for targeting construct*

Segment	Primer	Sequences
5′nHR	5P5PN	5′-GCG GCC CGT GGA GGA GTA TGA CAT CTC TGC TCT CC-3′
	5P3P	5′-CCG CTC GAG CGG AGG CTT CAC CCT CCA GTT GGA AGG AAC-3′
3′aHR	5BamHI	5′-GGA TCC CAA GGA TGA CAA TGT GAG CTA CGC-3′
	8XhoI	5′-AAT AAT GTA ACT CGA GTC ACG ATG ATC ACC-3′
3′bHR	7XhoI	5′-TCA TCG TGA CTC GAG TTA CAT TAT TTC TCG-3′
	13KpnI	5′-ATG GGA CCT CTA TCT CCA GGG AAA GTC TGC-3′

in controls and that this can be used as a prognostic marker of HIV progression. 2B4 signaling requires a functional signaling lymphocytic activation molecule (SLAM)[5]-associated protein (SAP), as was shown by studies of NK cells from patients with X-linked lymphoproliferative disease (XLPD) (26–30). SAP is an adaptor molecule that has a single Src homology 2 domain and a short tail. This Src homology 2 domain binds specifically to the motif T-I-pY-x-x-V/I (31). The cytoplasmic domain of 2B4 contains four similar tyrosine motifs (T-x-Y-x-x-V/I) (18), and SAP has been shown to associate with 2B4 (19). Patients with XLPD have mutations in the SAP gene that prevent binding to 2B4. When 2B4 on these NK cells interacts with CD48 on EBV-infected cells, an inhibitory signal is sent, instead of the activating signal when functional SAP is present (27). As a result, the EBV infection cannot be controlled, contributing to the pathogenesis of XLPD.

To study the in vivo role of 2B4, we generated mice deficient in the expression of both isoforms of 2B4. Studies of 2B4-deficient mice, cited in this manuscript, suggest a complex role of 2B4 in rejecting B16 melanoma cells. Compared with $CD48^-$ melanoma cells, $CD48^+$ cells are poorly rejected by wild-type mice, suggesting that the expression of CD48 on tumor cells inhibits B16 cell killing. This is supported by the enhanced resistance to $CD48^+$ melanoma cells in $2B4^{-/-}$ male mice. However, $2B4^{-/-}$ female mice have poor resistance against both $CD48^+$ and $CD48^-$ melanoma cells, revealing a gender-specific role of 2B4 that is independent of CD48 expression on tumor cells. In vitro killing assays and in vivo depletion of NK cells using NK1.1 mAb indicated that interaction of NK cells with other immune cells may play a role in the generation of gender specificity.

Materials and Methods

Mice

All experimentation was conducted in accordance with institutional and governmental policies. Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. All animals were housed in a specific pathogen-free facility.

Cell lines and Abs

The mouse melanoma cell line B16 and the lung cancer cell line 3LL were maintained in RPMI 1640 medium supplemented with 10% FBS (HyClone), 10 mM HEPES, 10 mM nonessential amino acids, 100 U/ml penicillin, 100 U/ml streptomycin, and 1 mM sodium pyruvate (Invitrogen Life Technologies). Cells were maintained at 37°C in 5% CO_2. All Abs were purchased from BD Pharmingen unless stated otherwise. The anti-NK1.1 mAb clone PK136 was obtained from American Type Culture Collection. Ab was isolated by affinity purification on a protein A column.

Generation of the targeting construct

Genomic DNA prepared from C57BL/6 mice was used as a template for PCR to amplify three products, referred to as 5′nHR, 3′aHR, and 3′bHR, using the primers indicated in Table I. These three fragments were TA subcloned into pGEM-T Easy vector (Promega), and the constructs were verified by sequencing at the core facility, University of Texas Southwestern Medical Center. 5′nHR was subcloned into the vector pPNT (32) using *Not*I and *Xho*I restriction sites. The insert 3′aHR was digested with *Bam*HI and *Xho*I, and 3′bHR construct was digested with *Xho*I and *Kpn*I. Inserts were gel-purified using Qiagen gel extraction kit. Vector 5′nHRpPNT was digested with *Bam*HI and *Kpn*I and gel-purified. A three-way ligation was performed with the vector 5′nHRpPNT, digested with *Bam*HI and *Kpn*I and the two inserts (3′aHR and 3′bHR). After transformation, bacterial colonies were PCR-screened for correct ligation, and the targeting construct was verified by sequencing.

Culture and selection of recombinant mouse embryonic stem (ES) cells

The targeting construct was linearized with *Not*I and electroporated into the ES cell line Bruce 6 of C57BL6/J origin (33). ES cell culture and selection were performed as previously described (33). Surviving colonies were screened for homologous recombination by PCR as previously described using the primers listed in Table I.

Mouse screening

ES cell clones that were positive for homologous recombination were used for blastocyst injection. One clone (2H10) that was injected resulted in a chimera whose litter contained pups with germline transmission of the ES cell genotype. These heterozygous mice were bred, and genomic tail DNA of the progeny was screened by PCR to identify mice homozygous for the targeted 2B4 gene.

Flow cytometry

Thymocytes, splenocytes, and PBMC from C57BL6/J and $2B4^{-/-}$ mice (8–12 wk) were analyzed by flow cytometry using a panel of FITC- and PE-conjugated mAbs. Single cell suspensions were made by crushing the solid organs between the frosted parts of two microscope slides. RBC were lysed by hypotonic lysis using sterile water. For staining of PBMC, blood was anticoagulated using EDTA. Cells were stained with the panel of mAbs, then the RBCs were lysed using the Whole Blood Lysing Reagents kit (Coulter) according to the manufacturer's instructions. Stained cells were analyzed on a Coulter EPICS XL-MCL flow cytometer. For studying the NK cell receptor repertoire, splenocytes from wild-type or $2B4^{-/-}$ mice were triple color-stained for CD3, NK1.1, and the receptor of interest. Cells were gated on the $NK1.1^+/CD3^-$ population, and the expression of the third receptor was analyzed.

Mouse tumor model

B16 melanoma cells were injected i.v. through the lateral tail vein into syngeneic C57BL6/J or $2B4^{-/-}$ mice. Each experimental group consisted of five to seven mice that were 8–12 wk old. B16 cells were washed with PBS and resuspended in PBS to a density of 2×10^6 cells/ml. Each mouse was injected with 0.2 ml of cells (400,000 cells). Mice were killed 14 days later by CO_2 asphyxiation, and the lungs were removed. Metastatic colonies were visualized as black spots and counted. NK cells were depleted by injecting 200 μg of NK1.1 Ab i.p. 3 days and 1 day before injecting melanoma cells. This achieved a >97% depletion of $NK1.1^+$ cells from the spleen. NK-depleted animals were injected with 20,000 B16 cells each in a volume of 200 μl. Injecting 400,000 cells/NK-depleted mouse filled the entire lungs with tumors and made enumeration difficult in all groups of mice.

Transfection of B16 cells with murine CD48

Mouse CD48 cDNA was isolated from CTLL-2 cells by RT-PCR and subcloned into the *Eco*RI and *Xba*I sites of the mammalian expression

[5] Abbreviations used in this paper: SLAM, signaling lymphocytic activation molecule; SAP, SLAM-associated protein; ES, embryonic stem; HR, homologous recombinant; LAK, lymphokine-activated killer; XLPD, X-linked lymphoproliferative disease.

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vector pCI-Neo (Promega). Construct was confirmed by sequencing at Seqwright. B16 cells were stably transfected with the linearized vector by electroporation. Cells were selected in medium containing 1 mg/ml G418 (Sigma-Aldrich). Positive clones were analyzed for CD48 expression by flow cytometry using FITC-labeled anti-CD48 Ab (BD Pharmingen).

^{51}Cr release assay

^{51}Cr release assay was performed as described previously (34). Briefly, target B16 cells were labeled with ^{51}Cr for 1 h at 37°C. Labeled B16 cells were incubated with lymphokine-activated killer (LAK) cells at the indicated ratios for 4 h. Supernatants were collected, and the percent specific lysis was calculated using standard methods. LAK cells were generated by first enriching NK cells from mouse splenocytes by passing the splenic cell suspension through a nylon wool column, followed by magnetic depletion of $CD3^+$ cells. The enriched NK cells were cultured in complete RPMI 1640 with 1000 U/ml human rIL-2 (National Institutes of Health). On day 3 after the onset of culture, half the medium was replaced with fresh medium containing IL-2. Day 7 LAK cells were used in the cytotoxicity assay.

Statistical analysis

Statistical analysis was performed using Student's t test for two samples with equal variance. A value of $p \leq 0.05$ was considered significant.

Results

Generation of 2B4-deficient mice

The 2B4 targeting construct was designed to disrupt the expression of both splice variants, 2B4-S and 2B4-L. As shown in Fig. 1*A*, homologous recombination is expected to replace all of exon 2 and part of exon 3 of the 2B4 gene with the PGK-neo^r gene, therefore resulting in no 2B4 expression (6). An alternative possibility is that differential splicing from exon 1 to exon 4 might occur, resulting in a defective, truncated protein with the extracellular V and C domains removed. Of the 338 ES cell clones analyzed by PCR and Southern screen, eight were positive for homologous recombination (2.4%). Three clones were used for blastocyst injection and oviduct transfer. Clone 2H10 resulted in a chimera that transmitted the mutation through the germline. These chimeras were backcrossed onto albino C57BL/6 female mice, and the heterozygous pups were intercrossed. Progeny was screened by PCR of the tail genomic DNA and homozygous mutant mice were identified (Fig. 1*B*). Homozygous mutant mice were also analyzed for expression of the 2B4 or alternative truncated transcript. By RT-PCR of splenocyte mRNA, we found that the knockout mice do not express any 2B4 transcript (data not shown). To confirm the absence of 2B4 protein expression, splenocytes from wild-type and $2B4^{-/-}$ mice were analyzed by flow cytometry. Some 14.3% of wild-type splenocytes stained with anti-2B4 mAb, whereas the $2B4^{-/-}$ splenocytes failed to do so (Fig. 1*C*), indicating the absence of 2B4 cell surface expression.

Lymphoid development in $2B4^{-/-}$ mice

$2B4^{-/-}$ mice develop normally and appear healthy at least up to 10 mo of age. We analyzed the percentage of different cells in the PBMC, spleen, and thymus by flow cytometry as an indicator of lymphoid development in these mice. The expression profile of CD3, CD4, CD8, Cd11b, CD19, and NK1.1 in the PBMC and splenocytes of $2B4^{-/-}$ mice did not differ significantly from that in wild-type mice (Fig. 2). The female $2B4^{-/-}$ thymi, however, showed a slight, but consistent, increase in the immature $CD4^-/CD8^-$ cells compared with wild-type female thymi (Fig. 3*A*). This increase in immature thymocytes was not seen in the male $2B4^{-/-}$ mice (Fig. 3*B*). Whether this affects the function of T cells in $2B4^{-/-}$ mice remains to be seen. Also, whether this gender difference in thymocyte development plays a role in the gender-specific rejection of B16 cells is not known.

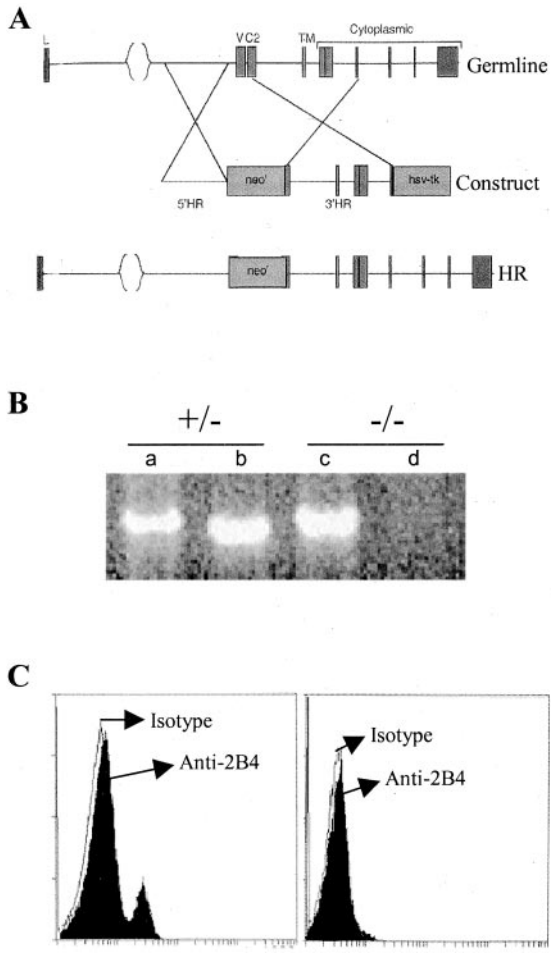

FIGURE 1. Targeted disruption of the 2B4 gene by homologous recombination. *A*, Experimental strategy. *Upper panel*, Organization of 2B4 gene (labeled germline). The rectangles indicate exons representing various domains of 2B4. L, Leader sequence; V, variable domain; C2, constant domain; TM, transmembrane domain; cytoplasmic, cytoplasmic domains. *Middle panel* (labeled construct), Targeting construct with the relative positions of 2B4 sequences, neomycin (neo) gene and the thymidine kinase (hsv-tk) cassette indicated. 5′HR and 3′HR are the two 2B4 sequences where homologous recombination is expected to occur. The crosses between the *upper* and *middle panels* indicate the regions where homologous recombination is expected to take place. The *lower panel* is the HR. The neomycin resistance gene replaces the V domain and part of C2 domain in the HR. *B*, PCR screen of mouse tail genomic DNA. Genomic DNA isolated from tail biopsies was screened by PCR. Each mouse was screened by two PCRs. The first lane for each mouse (*lanes a* and *c*) is PCR with a 5′ primer specific for the 2B4 gene (upstream of site of homologous recombination) and a 3′ primer specific for the neomycin resistance region of targeting construct. Amplification from this pair of primers indicates the presence of homologous recombination in the genome of the mouse. The second lane for each mouse (*lanes b* and *d*) is PCR using the same 5′ primer, but the 3′ primer is 2B4 specific in the region deleted by homologous recombination of the construct. Amplification from this pair of primers indicates the presence of an intact 2B4 gene in the genome. +/−, Heterozygous mutant; −/−, homozygous mutant. *C*, Expression of 2B4 in wild-type and $2B4^{-/-}$ mice. Splenocytes from wild-type (*left*) or $2B4^{-/-}$ (*right*) mice were stained with FITC-conjugated anti-mouse 2B4 Ab and analyzed by flow cytometry. □, Cells stained with isotype control; ■, cells stained with FITC-labeled anti-2B4 Ab. More than 20 mice from different litters were used for staining the splenocytes. The results shown are representative.

Receptor repertoire on $2B4^{-/-}$ NK cells

All murine NK cells express 2B4. Indeed, 2B4 is expressed very early in NK cells development, before acquisition of other receptors (M.

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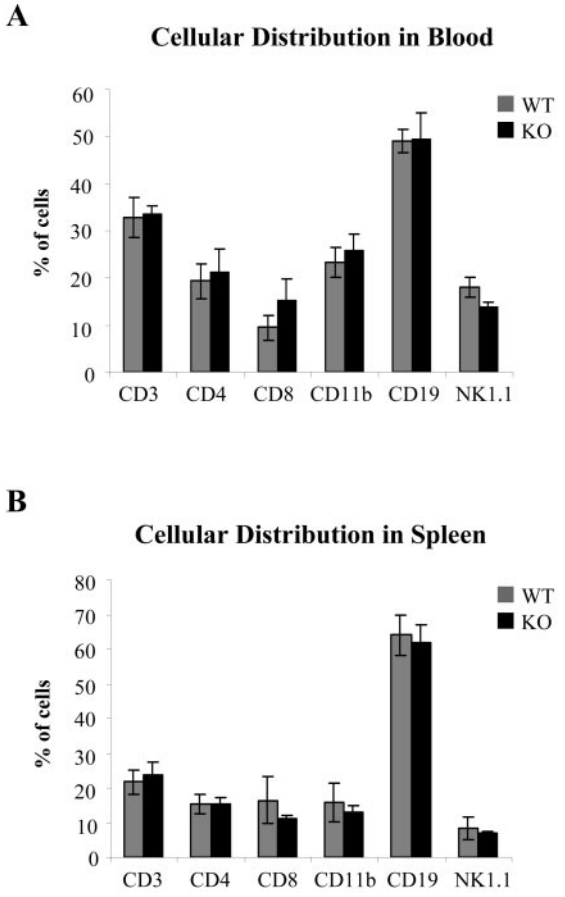

FIGURE 2. Cellular distribution in the blood and spleen of 2B4$^{-/-}$ mice. *A*, Blood was anticoagulated using EDTA, and cells were stained for various cell surface markers. RBCs were lysed, and samples were analyzed by flow cytometry. Error bars in the bar graph indicate the SD. Results are for four wild-type and four 2B4$^{-/-}$ mice. WT, wild type; KO, 2B4$^{-/-}$ mice. *B*, Splenocytes were purified as explained in *Materials and Methods* and were stained for various cell surface markers. Error bars in the bar graph indicate the SD. Results are for four wild-type and four 2B4$^{-/-}$ mice.

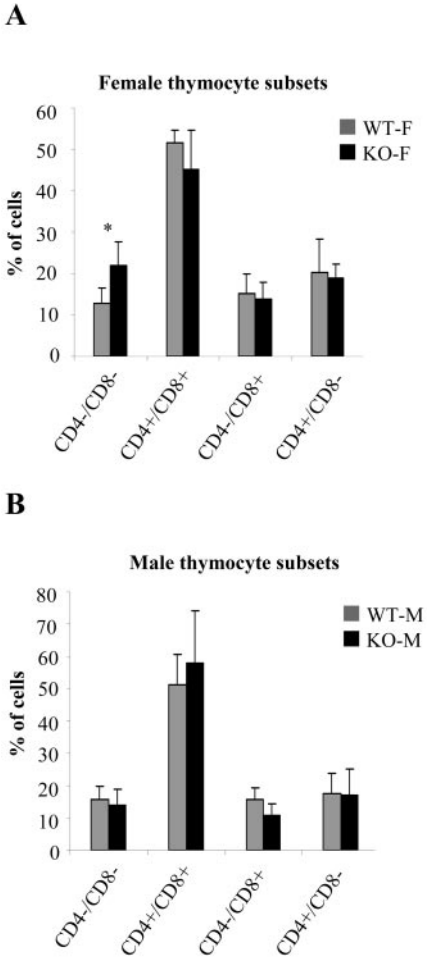

FIGURE 3. Lymphoid development in the thymus of 2B4$^{-/-}$ mice. Thymocytes were isolated from wild-type and 2B4$^{-/-}$ mice and double color-stained for CD4-FITC and CD8-PE. *A*, T cell subsets in the female thymi. Error bars indicate the SD. *, $p < 0.05$. *B*, T cell subsets in the male thymi. Error bars indicate the SD. Each group consists of at least five animals. □, Wild type; ■, 2B4$^{-/-}$ mice.

McNerney and V. Kumar, unpublished observations). To study whether the absence of 2B4 affects the development of NK cells, we looked at the expression of various NK cell receptors. Except for CD2 and CD48, which are expressed on all NK cells, other receptors analyzed were expressed on subsets of NK cells. By flow cytometry we analyzed the expression pattern of these receptors. As shown in Fig. 4, 2B4$^{-/-}$ NK cells have a receptor repertoire that does not differ from that of the wild-type NK cells. The mean fluorescence intensity of either of these receptors did not differ significantly between the wild-type and knockout groups, suggesting identical levels of expression of the surface molecules.

CD48-transfected B16 cells form more pulmonary tumors in both wild-type male and female mice

To study the in vivo role of 2B4, we used a mouse tumor model in which syngeneic B16 melanoma cells are injected i.v., and pulmonary metastases are enumerated 14 days later. The two isoforms of 2B4, 2B4-S and 2B4-L, that have been reported to function as activating and inhibitory receptors, respectively, are both expressed in bulk LAK cell culture. It is not known whether individual LAK cells express both isoforms or different subsets express one or the other form. It is also not known whether one or the other isoforms is the dominant receptor. Toward this end, we stably transfected B16 cells with murine CD48 (Fig. 5*A*). Wild-type male and female mice were injected with CD48$^-$ and CD48$^+$ B16 cells. As shown in Fig. 5*B*, there was a significant increase in the number of tumors of CD48$^+$ B16 cells compared with CD48$^-$ B16 cells in both male and female mice. Male mice had, on the average, 163 ± 71 tumors of CD48$^-$ B16 compared with 406 ± 61 of B16-CD48 ($p = 0.0004$). Female mice had 181 ± 65 tumors of CD48$^-$ B16 compared with 423 ± 30 of B16-CD48 ($p = 0.0001$). Although previous in vitro studies have shown an activating role of 2B4, this tumor experiment suggests that 2B4-CD48 interaction is inhibitory. This is in agreement with our recent study that demonstrates an inhibitory role for 2B4 (34). Another study confirms the inhibitory role of 2B4-CD48 interaction and shows that this inhibitory function of 2B4 is independent of SAP (35). If this conclusion is correct, one might expect 2B4$^{-/-}$ mice to better reject the CD48$^+$ B16 melanoma cells. Indeed, 2B4$^{-/-}$ male mice had significantly fewer tumors from CD48$^+$ cells compared with wild-type mice (compare Fig. 5, *B* and *C*). Surprisingly, however, female 2B4$^{-/-}$ mice had similar number of tumors as 2B4$^{+/+}$ female mice. Furthermore, female 2B4$^{-/-}$ mice had poor resistance to CD48$^-$ cells. Thus, it appears that female 2B4$^{-/-}$ mice have impaired ability to reject B16 cells regardless of the expression of CD48 on tumor cells.

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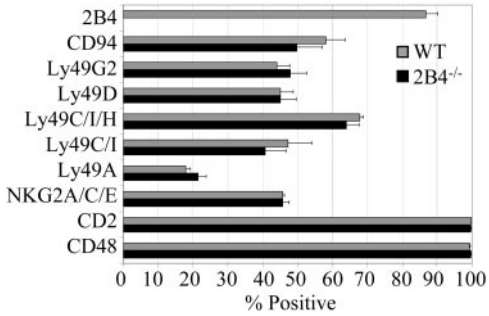

FIGURE 4. NK receptor repertoire. Splenocytes were isolated from wild-type and $2B4^{-/-}$ mice. Cells were triple color-stained for CD3, NK1.1, and the receptor to be analyzed. Gating was performed on the $NK1.1^{+}/CD3^{-}$ cells, and the expression of the third receptor was analyzed. □, Wild type; ■, $2B4^{-/-}$ mice.

Female $2B4^{-/-}$ mice have reduced ability to resist $CD48^{-}$ tumors

Because the female $2B4^{-/-}$ mice are deficient in the ability to resist the growth of $CD48^{-}$ melanoma cells, we analyzed the development of $CD48^{-}$ B16 tumors in groups of male and female, wild-type and $2B4^{-/-}$ mice. Whereas male $2B4^{-/-}$ mice had fewer tumor nodules compared with wild-type controls (average, 71 ± 38 vs 163 ± 71; $p = 0.03$), female $2B4^{-/-}$ mice had ~3 times as many tumors as wild-type controls (average, 519 ± 152 vs 181 ± 65; $p = 0.0013$; Fig. 6). This suggests a gender-specific role for 2B4 in host defense mechanisms against B16 melanoma. We repeated the experiment using the mouse lung cancer cell line, 3LL. This cell line also does not express CD48. We did not find any gender-specific difference in $2B4^{-/-}$ mice (data not shown). In fact, the number of 3LL tumors in the lungs of knockout mice (regardless of the gender) did not differ from that in wild-type mice. This indicates that the gender-specific effect in the deficient mice might be unique to B16 melanoma cells.

Complex role of NK cells in the gender-specific rejection of B16 melanoma cells

To gain insight into the mechanism of gender specificity, we studied whether NK cells contribute to this effect. We compared the cytotoxicity of wild-type and knockout, male and female LAK cells in a ^{51}Cr release assay using $CD48^{-}$ B16 cells as the target. As shown in Fig. 7*A*, the cytotoxicity of knockout male LAK cells did not differ from that of knockout female LAK cells. This suggests that NK cells may not play a role in the mechanism of gender specificity. The knockout LAK cells as a group, however, had significantly reduced cytotoxic potential compared with wild-type LAK cells. This is in keeping with the proposition that during activation of NK cells, NK-NK interactions via 2B4-CD48 ligation stimulate NK cells. We also analyzed the role of NK cells in the in vivo model. Wild-type and knockout mice were depleted of NK cells using the anti-NK1.1 Ab. These mice were then challenged with $CD48^{-}$ B16 cells. Surprisingly, NK-depleted, knockout mice did not exhibit the gender specificity (Fig. 7*B*) of nondepleted mice, indicating a role for NK cells in production of the gender-specific effect. This apparent discrepancy between the in vitro and in vivo results can be explained on the basis of a complex interaction between NK cells and other immune cells, possibly T cells, resulting in the gender-specific effect. The ^{51}Cr release assay, being an isolated system, fails to appreciate the effects of interaction among different immune cells.

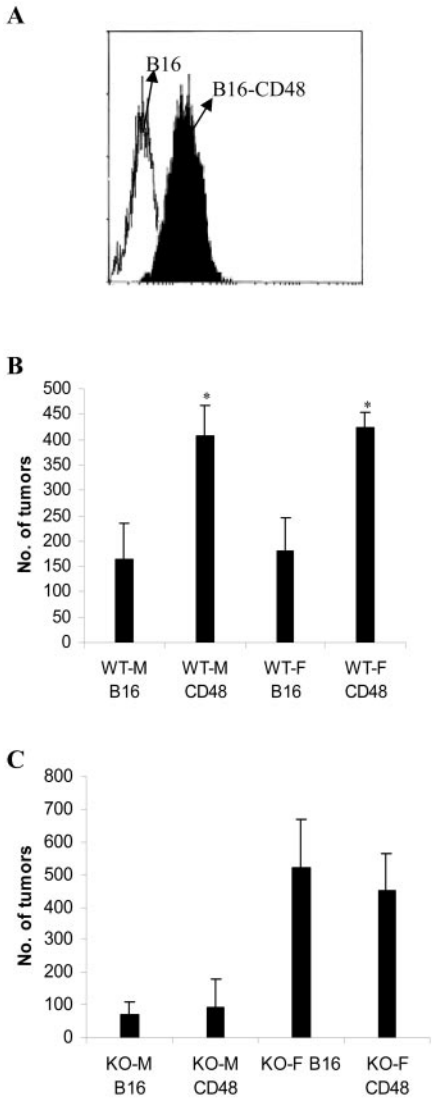

FIGURE 5. Growth of B16 and B16-CD48 melanoma cells. *A*, B16 cells were stably transfected with pCI-Neo-CD48 construct. Transfected cells were selected and analyzed by flow cytometry for the expression of surface CD48 using an FITC-labeled anti-CD48 Ab. □, B16 untransfected; ■, B16 transfected with murine CD48. *B*, Comparison of the number of B16 and B16-CD48 tumors in the lungs of wild-type male and female mice. Four hundred thousand cells were injected i.v. in each mouse, and tumors were enumerated 14 days later. Error bars indicate the SD. ∗, Statistical significance ($n \geq 5$). B16, Untransfected cells; B16-CD48, CD48 transfected cells. *C*, Comparison of the number of B16 and B16-CD48 tumors in the lungs of $2B4^{-/-}$ male and female mice.

Discussion

In the present study we have generated and characterized mice deficient in 2B4 expression by gene targeting. Although lymphocyte development is largely normal, $2B4^{-/-}$ mice show an increase in immature $CD4^{-}/CD8^{-}$ cells in the thymus only in females. The significance of this finding with regard to T cell function or the gender-specific rejection of B16 cells remains to be seen. CD48-deficient mice demonstrated a developmental defect in the thymus and a profound defect in the activation of CD4 T cells through the TCR (36). Before CD48 was identified as the ligand for 2B4, CD2 was recognized as the CD48 ligand. Because CD2-deficient mice are phenotypically normal (37), a thymic developmental defect in 2B4-deficient mice is not surprising. Recently, it was shown that NK cells can eliminate developing MHC class

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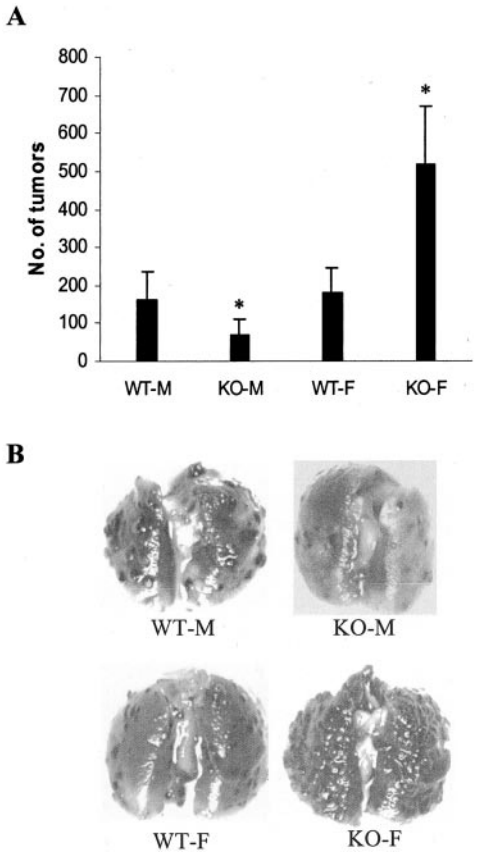

FIGURE 6. Growth of B16 melanoma cells in wild-type and 2B4$^{-/-}$ mice. *A*, Comparison of the number of B16 tumors in the lungs of wild-type and 2B4$^{-/-}$ mice. Four hundred thousand B16 cells were injected i.v. in each mouse, and tumors were enumerated 14 days later. Error bars indicate the SD. *, Statistical significance ($n \geq 5$). *B*, Representative lung pictures from wild-type and 2B4$^{-/-}$ mice. ●, Melanoma tumors; WT-M, wild-type male; WT-F, wild-type female; KO-M, 2B4$^{-/-}$ male; KO-F, 2B4$^{-/-}$ female.

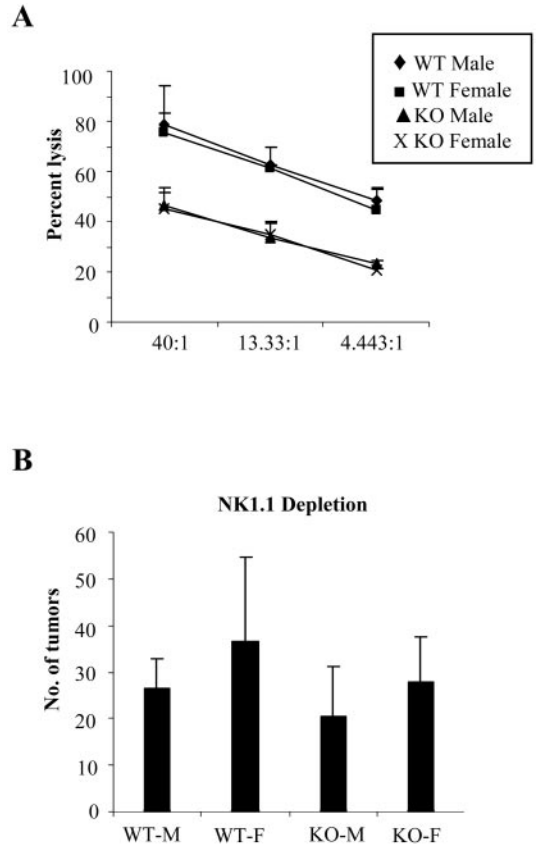

FIGURE 7. Complex role of NK cells in the gender-specific rejection of B16 cells. *A*, In vitro cytotoxicity of LAK cells against B16 cells as described in *Materials and Methods.* Briefly, labeled B16 cells were incubated with LAK cells from wild-type male (♦), wild-type female (■), knockout male (▲), and knockout female (X) mice in triplicate at the E:T cell ratio indicated on the *x*-axis. Supernatants were analyzed for percent specific lysis. Error bars indicate the SD. *B*, Growth of B16 cells in the lungs of NK-depleted mice. NK cells were depleted from mice using anti-NK1.1 mAb. Twenty thousand B16 cells were injected in each NK-depleted mouse. Lungs were harvested 14 days later, and the number of B16 tumors was enumerated. Error bars indicate the SD. Each group consists of at least seven mice.

I-deficient thymocytes (38). This leads to a delayed development of thymocytes. However, MHC class I-deficient CD8 T cells do develop and are functional. This argues for a possible regulatory role of NK cells in the development of thymocytes.

To study the physiological role of 2B4 in tumor rejection, we used an experimental tumor metastasis model, challenging the mice with syngeneic B16 melanoma cells. When wild-type mice were challenged with CD48$^+$ and CD48$^-$ B16 melanoma cells, the CD48$^+$ B16 cells produced many more tumors compared with CD48$^-$ cells. This difference was seen regardless of sex, and it indicates that the interaction between 2B4 and CD48 inhibits the antitumor immune response. This conclusion is clearly supported by the enhanced ability of male 2B4$^{-/-}$ mice to clear CD48$^+$ B16 cells more efficiently than wild-type male mice. In contrast, it appears that female 2B4$^{-/-}$ mice are poor at controlling tumor growth regardless of whether the tumor expresses CD48 (Figs. 5*C* and 6*A*). This indicates that in female 2B4$^{-/-}$ mice there is an impairment of B16 melanoma rejection that is independent of target cell expression of CD48. Because B16 cells do not express CD48, the cognate receptor for 2B4, one would not expect to see differences in tumor rejection between 2B4$^{-/-}$ and wild-type mice. However, recent studies suggest that 2B4-CD48 interaction on neighboring T cells can modulate the function of these cells and hence their action on target cells (15, 16). Similarly, 2B4-CD48 interaction among NK cells have also been found to activate NK cells.[4] In view of these observations, 2B4-CD48 interactions could regulate host resistance against tumor by two distinct mechanisms: interaction of NK cell 2B4 with tumor-associated CD48 and 2B4-CD48 interactions among NK cells. The data presented in this study shows the likely effects of both such interactions.

To determine the cell type that plays a role in this gender effect, we performed in vitro ^{51}Cr release assay using LAK cells from wild-type and knockout mice of both genders as effecter cells. Male and female knockout LAK cells had similar cytotoxicity against B16 cells, indicating that female NK cells do not have an intrinsic defect. This supports the possibility of a role for non-NK cells, such as T cells, in this effect. In vivo, we saw no gender difference in the growth of B16 tumors in NK-depleted mice. This suggests that NK cells contribute to the gender difference seen in the 2B4$^{-/-}$ mice. There are increasing data demonstrating NK-T cell interaction. In a recent study Assarsson et al. (17) have shown that interaction between 2B4 on NK cells and CD48 on T cells enhances the proliferation of T cells in response to anti-CD3 and specific Ag as well as increases the fraction of CD69$^+$ T cells after anti-CD3 cross-linking. Markovic et al. (39) have shown that CD8 T cells regulate IFN-mediated NK cell activity against B16 melanoma cells in vivo. Another study demonstrated that NK cells

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play a crucial role in generation of the CTL response against B16 melanoma cells (40). Recently, Xu et al. (41) have shown a synergistic interaction between NK cells and CD8 T cells in rejecting B16 melanoma in response to IL-12 treatment. All these studies indicate that interactions between the different components of immune system play an important role in the rejection of cancer cells. The gender-specific difference we see in 2B4$^{-/-}$ mice could be the result of an interaction between NK and other immune cells.

This gender-specific effect in 2B4$^{-/-}$ mice was not seen with the mouse lung cancer cell line 3LL (data not shown). Thus, the gender-related effect appears to be specific to B16 melanoma cells. If this is indeed the case, it suggests that an Ag-specific T cell response could play a role in producing this effect in a 2B4-dependent manner. A number of previous studies have shown that T cell responses are important in the rejection of B16 melanoma cells in mice (42–47). Melanoma-specific Ags have been identified that can induce a T cell response. Some of these include tyrosinase-related protein-1 and tyrosinase-related protein-2 in the mouse and melanoma/melanocyte Ag recognized by T cells-1, gp100, melanoma-associated Ag-1, and melanoma-associated Ag-3 in humans (45, 48–50). A gender-related defect in 2B4$^{-/-}$ T cells could be a sex hormone-based mechanism. The female hormone, estrogen, is known to regulate the development and function of immune cells, including T cells (51–60). Estrogen might alter the MAPK cascade in immune cells, because it can induce the MAPK pathway in murine cerebral cortical cells (61, 62). This might lead to a functional interaction between the estrogen pathway and the cytotoxicity receptors, because PI3K plays a pivotal role in the cytotoxicity of NK cells (63). Interestingly, we have shown that NK cell cytotoxicity stimulated through 2B4 is dependent on the MAPK pathway (64). Whether 2B4 signals through the MAPK pathway in T cells also is not known. Cross-talk at the molecular level between the 2B4 receptor and sex steroids appears possible considering that some cytokines have an intimate cross-communication with steroid hormones (65).

There are several other examples of gender-based differences in immune regulation. Systemic lupus erythematosus is an autoimmune disease in humans that predominantly affects females. Linkage analyses have shown strong association of the disease with the 1q21–44 region of the genome (66). A murine model of systemic lupus erythematosus (NZM2410 strain) also shows strong linkage with the homologous region of chromosome 1 (67). The susceptibility region has been termed *sle1*, and B6.*sle1*, a congenic strain that has the NZM susceptibility region on a C57BL/6 background, has been shown to have a loss of tolerance to chromatin due to the *sle1* region introduced in this strain (68, 69). Fine mapping of this region identified three loci, *sle1a*, *sle1b*, and *sle1c*, that could contribute to the loss of tolerance to chromatin (70). Although all three loci had a higher penetrance in female mice, only the *sle1b* locus showed a significant gender difference. Interestingly, the mouse 2B4 gene is in the region of *sle1b*. Because 2B4$^{-/-}$ mice show a gender-specific alteration in immune function, it will be interesting to investigate whether the 2B4 polymorphism contributes to the autoimmune susceptibility of the *sle1b* locus. If this is true, it suggests that 2B4 regulates the male and female immune systems differently. This has important implications in autoimmune disorders, which tend to be predominant among females.

In summary, our study suggests that 2B4 might be involved in the normal development of thymocytes. The tumor experiments show that 2B4 plays an in vivo role in the rejection of tumor cells. Mice receiving CD48-transfected B16 cells had more pulmonary metastasis, indicating that the 2B4-CD48 interaction might be inhibitory in vivo. Because, tumor rejection is altered in a gender-specific manner in 2B4$^{-/-}$ mice, 2B4 might be playing a different role in the two sexes. This has implications in many immune disorders, especially autoimmune diseases that show a gender bias. Our study warrants further investigation of the gender-specific role of 2B4.

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