Resistance of novel mouse strains different in MHC class I and the NKC domain to the development of experimental tumors

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Abstract. To elucidate the immunological mechanisms critical for tumor progression, we bred novel mouse strains, different in the NKC and H-2D domains. We used inbreeding to generate hybrids of Balb/c and C57BL/6 of stable H-2Db+d-NK1.1neg and H-2Db-d+NK1.1high phenotypes. We analyzed the growth of three established MHC class I-deficient tumor cell lines: TC-1/A9 tumor (HPV-associated) and B16F10 melanoma, both syngeneic to C57BL/6, and the MCB8 (3-methycholanthrene-induced tumor) syngeneic to Balb/c. Furthermore, we induced colorectal carcinoma by azoxymethane-DSS treatment to test the susceptibility to chemically-induced primary cancer. We found that the novel strains spontaneously regressed the tumor transplants syngeneic to both Balb/c (MCB8) and C57BL/6 (B16F10 and TC-1/A9) mice. The H2-Db+d-NK1.1neg, but not the H2-Db-d+NK1.1high strain was also highly resistant to chemically-induced colorectal cancer in comparison to the parental mice. The immune changes during TC-1/A9 cancer development involved an increase of the NK cell distribution in the peripheral blood and spleen along with higher expression of NKG2D activation antigen; this was in correlation with the time-dependent rise of cytotoxic activity in comparison to C57BL/6 mice. The TC-1/A9 cancer regression was accompanied by higher proportion of B cells in the spleen and $B220^+/CD86^+$ activated antigen-presenting B cells distributed in the lymphoid organs, as well as in the periphery. The changes in the T-cell population were represented mainly by the prevalence of T helper cells reflected by grown CD4/CD8 ratio, most prominent in the b+d-NK1.1neg strain. The results of the present study imply usefulness of the two novel mouse strains as an experimental model for further studies of tumor resistance mechanisms.

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Introduction

Carcinogenesis, a multistage process, during which DNA mutations as well as epigenetic changes accumulate in the cell, is promoted in individuals with genetic susceptibility, hormonal disturbances or unfavorable immunological background (e.g. presence of chronic inflammation) (1-3).

The antitumor immune response is based on tight co-operation between the components of the innate and adaptive immunity, and is strongly influenced by the active role of the tumor microenvironment manifested by immune cell suppression, selection of non-immunogenic tumor cell variants (4). Natural killer (NK) cells are primarily involved in the surveillance of major histocompatibility complex class I (MHC I)-deficient tumors, but also participate in priming of the specific, MHC I-restricted immune responses via interferon-gamma (IFN-γ) secretion leading to upregulation of MHC I expression on tumors, potentiating cytotoxic T lymphocyte (CTL)-mediated lysis (5,6).

The outcome of NK-target cell interaction (killing or not killing) is governed by integration of signals from numerous receptors, some activating, others inhibitory. The NK cell receptor genes are organized in two main clusters, the leukocyte receptor complex and the natural killer gene complex (NKC), located on different chromosomes. The receptors of the NKC locus manifest genetic polymorphism that includes both allelic variations within a single gene and structural genomic variation of total gene content (7,8). The NKC on distal chromosome 6 contains several clusters of genes encoding NKR-P1, Ly49, CD94 and NKG2 receptor families (9,10). Most genes of the NKC are conserved across species, with orthologues identified in mouse and human genome (11). The number of Ly49 genes varies between individual mouse strains, and there is also evidence for extensive allelic polymorphism, accompanied by distinct H-2 ligand specificities and affinities. In C57BL/6 mice, the Ly49 family consists of 15 genes encoding activating Ly49D and H, and inhibitory Ly49 Q, E, F, I, G, J, C and A receptors, whereas BALB/c

mice possess the smallest Ly49 haplotype containing 6 functional inhibitory receptor genes (*Ly49q*, *e*, *i*, *g*, *c* and *a*) and a single activating receptor gene (*Ly49l*) (12). The NK cell receptor protein 1 (NKR-P1) is present in several isoforms that are encoded by *Nkr-p1a*, *b*, *c*, *d* and *f* genes (*Nkr-p1e* and *Nkr-p1g* are probably pseudogenes with no functional transcript). C57BL/6 mice possess *Nkr-p1d*, whereas Balb/c mice *Nkr-p1b*. NKR-P1C receptor isoforms differ between C57BL/6 and Balb/c mice in the amino acid sequence and only NKR-P1C^{B6} can be detected by anti-NK1.1 (PK136) antibody (13,14). While the physiological ligands for inhibitory NKR-P1B/D, and NKR-P1F were identified as C-type lectin-related (*Ocil*/*Clr*) molecules encoded in the NKC domain, the ligands of activation receptors NKR-P1A and C remain unknown. The Clr-b expression is downregulated upon cell stress caused by DNA damage or malignant transformation. The NKR-P1B-Clr-b interactions thus represent an alternative form of 'missing-self' recognition independent of H2-D molecules (14,15). NKR-P1B-deficient NK cells are more efficient in killing MHC-I-deficient cells, possibly due to a lack of Clr-b-mediated inhibition and/or a higher dependence on MHC-I-mediated education (16).

There are controversies in the role of NK cells in the immune response due to their ability to produce both anti-inflammatory (IL-10) and pro-inflammatory (IFN-γ) cytokines. NK1.1^{+} cells are known to contribute to antitumor immunity, prevent autoimmunity and GVHD (17-19). On the other hand, NK1.1^{+} cell depletion has been shown to decrease chronic exhaustion of T cells, and thus prevent tumor recurrence (20-26). In the present study, we investigated whether the NKC and H2-D polymorphism influences cancer development as well as the antitumor immune response. As there are neither mouse mutants with deleted NKR-P1C^{B6}, nor congenic C57BL/6 mice with NKR-P1C^{BALB} genes available, we crossed C57BL/6 (H-2Db+H-2Dd-NK1.1high) and Balb/c (H-2Db-H-2Dd+NK1.1neg) mice to generate two novel strains which possess NKC^{B6} domain on H-2D background of Balb/c origin (b-d+NK1.1high) and NKC^{BALB} on C57BL/6 H2-D background (b+d-NK1.1neg). In this study, we describe the basic characteristics of the newly generated strains on both pheno-genomic and immunological level. The phenotype and function of the tumor-specific cytotoxic effectors, as well as vulnerability of the new strains to the experimental tumor growth were followed.

Materials and methods

Experimental animals. Eight-week-old inbred female C57BL/6 and Balb/c (Charles River Laboratories, Munich, Germany) as well as newly generated mouse strains were housed under natural day/night conditions (22˚C, 55% relative humidity), and fed on a commercial ST1 diet (Velas, Prague, Czech Republic) *ad libitum*. The novel mouse strains were produced by inbreeding (F_{29}) of parental C57BL/6 (H2Db+H2Dd-NK1.1high) and Balb/c (H2Db-H2Dd+NK1.1neg) mice based on the NKC domain gene expression controlled by DNA analysis and H-2D cytometric phenotyping to obtain stable H2-Db-H2-Dd+NK1.1high (b-d+NK1.1high) and H2-Db+H2-Dd-NK1.1neg (b+d-NK1.1neg) phenotypes. The homozygosity in the NKC domain (NK1.1 expression) and the H2D haplotype of the newly established strains was examined by flow cytometry and PCR genotyping before mating. Breading of mice and all experimental procedures were conducted under SPF conditions in accordance with the European Convention for the Care and Use of Laboratory Animals as approved by the Czech Animal Care and Use Committee.

Tumor cell lines. The TC-1/A9 tumor cell line (27) was derived from TC-1 cell line (obtained from the ATCC Collection) developed by co-transfection of murine C57BL/6 lung cells with HPV16 E6/E7 genes and activated (G12V) by Ha-ras plasmid DNA (28). TC-1/A9 tumor cell subline, deficient in MHC class I molecules, escaped from the selection pressure mediated by the specific immune response. The MCB8 fibrosarcoma was induced by 3 methylcholanthrene in Balb/c mouse in the Laboratory of Tumor Immunology IMG ASCR, Prague and was maintained by serial subcutaneous *in vivo* passages in Balb/c mice (Bubeník *et al*, unpublished data). The stabilized MCB8 cell line was further cultivated *in vitro*. B16F10 mouse melanoma and YAC-1 lymphoma were obtained from the ATCC. All cells were maintained in RPMI-1640 medium (Sigma-Aldrich GmbH, Steinheim, Germany) supplemented with 10% FCS (PAN-Biotech GmbH, Aidenbach, Germany), 2 mM L-glutamine, and antibiotics and cultured at 37˚C in humidified atmosphere with 5% CO_2.

Tumor cell inoculation. Mice were injected subcutaneously (s.c.) with B16F10 ($1x10^5$ cells/mouse), TC-1/A9 (5x105 cells/mouse), or MCB8 tumor cells ($5x10^5$ cells/mouse), in 0.1 ml phosphate-buffered saline (PBS) into the lower back on day 0. For induction of lung metastases, the mice were injected with $2.5x10^4$ B16F10 cells into the tail vein. Mice were observed twice a week and the size of the tumors was recorded. The tumor dimensions (length and width) were measured with a caliper every 2-3 days until the controls reached the tumor volume maximum 5,000 mm^3. The tumor size was expressed as the tumor volume (mm^3) according to the formula: V=0.523 x (length of tumor) x (width of tumor)^2.

Induction of colorectal carcinoma (CRC). The CRC in all studied strains of mice was performed according to Nature Protocols (29). Based on our preliminary tests, the optimal non-toxic dose of azoxymethane (AOM) for parental C57BL/6 and Balb/c mice, inducing 100% incidence of CRC after 3 months, was 6.5 mg/kg intraperitoneally, and 2.5% (wt/vol) dextran sodium sulfate (DSS) solution in drinking water as an inflammatory agent 7 days after AOM injection for 5 consecutive days. The development of colorectal carcinoma was evaluated after 3 months from the beginning of the induction protocol. The presence or absence of rectal bleeding was followed regularly.

Isolation of spleen, lymph node and peripheral blood cells. Peripheral blood samples from the tail vein were drawn into heparinized medium, seeded into U-bottomed 96-well microtiter plates (Nunc, Roskilde, Denmark) and erythrocytes were lysed by using 0.15 M ammonium chloride buffer (22˚C, 12 min). The cells were centrifuged (400 g for 2 min), washed three times in ice-cold PBS and used for FACS analysis. Spleens and regional lymph nodes were dissected, squeezed

Table I. Primer sequences used for genotyping of NKC domain in novel mouse strains.

	Forward (5'→3')	Reverse (5'→3')
β-actin	AGAGGGAAATCGTGCGTGAC	ACGGCCAGGTCATCACTATTG
Nkr-p1a	ACAAGTAGGGGCTGTGATGG	CTGAAAACCCTGCTGAAAGC
Nkr-p1b	AGGGAGCAGGAAGAGAGGAC	AGTCTTGTGGGCACTCTAAA
Nkr-p1cBalb/c	GAAAATGGCAGCTGTGCCA	TGCTTTCAGAGTCCATGTGC
Nkr-p1cC57Bl/6	GAAAATGGCAGCTGTGCCT	TGCTTTCAGAGTCCATGTGC
Nkr-p1d	AGGGAGCAGGAAGAGAGGAC	AGTCTTGTGGGCACTCTAGC
Nkr-p1f	TCTGAAATCTGGCTGTGCTG	TGGGACTTTTGGGTTCTTTG
Ly49C	TTGTAGGCCAAGCAATGAAAC	TCAAGTTTAGATGGGCCATTG
Ly49D	AAAAAGCTCGCCTCAGAGTTC	CTCTGCCTGTGTGCTGTAAGT
Ly49F	GTGTTAAATACTGGTTCTGCTACCG	CTCTTGCAGCTTTGTCTCTATTCAC
Ly49H	TCTTCTTGGAGCCTCTTAGGG	GCCTGTGTCTCGTGAAGAATC
Ly49L	TAAGTGCAGCACCACCACTC	TGTCTGAAGGAACCAGGAGC
CD69	CCCTTGGGCTGTGTTAATAGTG	AACTTCTCGTACAAGCCTGGG
NKG2D	ACGTTTCAGCCAGTATTGTGC	GGAAGCTTGGCTCTGGTTC

through a nylon mesh, washed three times in H-MEMd medium (Sebac, Aidenbach, Germany), and used immediately for assays.

Cytotoxicity assay. The cytotoxic activity was estimated using the standard ^{51}Cr-release assay (30). Mouse spleen cells (effectors) were seeded in pentaplicates into round-bottomed 96-well microtiter plates (Nunc). Subsequently, 10^4 tumor targets (YAC-1 or TC-1/A9 cells) labelled for 90 min with $Na_2{}^{51}CrO_4$ were added at an effector:target (E:T) ratio of 50:1 and incubated for 18 h at 37˚C in humidified atmosphere containing 5% CO_2 (Jouan, St. Herblain, France). The cell-free supernatants were harvested (0.025 ml/sample), 0.075 ml of scintillation cocktail (SuperMix; Wallac, Turku, Finland) was added, and the radioactivity was measured by MicroBeta® TriLux scintillation counter (Wallac). The percentage of cytotoxicity was calculated according to the following formula: Cytotoxicity [%] = 100 x (experimental cpm - spontaneous cpm)/(maximum cpm - spontaneous cpm). For spontaneous and maximal release controls, tumor targets were cultured alone or with 10% Triton X-100 (Sigma-Aldrich), respectively.

PCR genotyping of NKC domain genes. Genomic DNA for genotyping was obtained from mouse tails. A total of 3 mm of mouse tails were incubated with lysis buffer containing 100 mM Tris-HCl, 5 mM EDTA, 0.5% SDS, 200 mM NaCl, and 4 μg Proteinase K per tail (Qiagen, Hilden, Germany) at 55˚C for 4 h. Homogeneous samples were boiled at 95˚C for 10 min, diluted by water and used as a template for PCR. Amplification was carried out with 0.5 U of HotStarTaq® DNA polymerase (Qiagen) and 400 nM primers in the iCycler5 instrument (Bio-Rad Laboratories, Hercules, CA, USA). Gene for β-actin was used as a load control. Primers for amplification are summarized in Table I.

Flow cytometry. Cell suspensions prepared from blood, spleens and lymph nodes of individual mice (as described above) were resuspended in PBS. The following monoclonal antibodies were used according to the manufacturer's protocol: H2-Db-FITC, H2-Dd-PE, NK1.1-APC (clone PK136), NkP46-FITC, NKG2D-biotin, CD45R/B220-BD-Horizon V500, CD3-BD-Horizon V450, CD4-PerCP, CD8-APC-eFluor780, CD49b (DX5)-FITC, CD69-PE-Cy7, CD86-APC (BD Biosciences, San Jose, CA, USA or eBioscience, San Diego, CA, USA). Biotin was stained by Qdot 605 streptavidin (Invitrogen, Grand Island, NY, USA). Samples were measured by BD LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) in a four-laser set-up (405, 488, 561 and 633 nm) and data were offline-compensated and evaluated based on single-stain controls in FlowJo version 9 (Tree Star, Inc., Ashland, OR, USA). Morphology and doublet exclusion was based on forward-scatter (FSC) area, FSC height and side-scatter (SSC) area; propidium iodide or Hoechst 33258 (BD Biosciences) was used for exclusion of non-viable cells.

Statistical analyses. Student's t-test and analysis of variance (ANOVA) from NCSS (Number Cruncher Statistical System, Kaysville, UT, USA) statistical package were used for statistical analyses of *in vitro* and *in vivo* experiments, respectively. P-values <0.05 were considered as significant ($P<0.05$; $P<0.01$; $P<0.001$).

Results

Characterization of newly generated mouse strains. To elucidate the participation of the NKC domain and H2D haplotype in cancer surveillance, the b+d-NK1.1neg and b-d+NK1.1high hybrids of Balb/c and C57BL/6 strains were generated by inbreeding. The NKC domain stability was further proved by PCR genotyping of *Nkr-p1* and *Ly-49* gene family polymorphism. The genes shared by Balb/c and C57BL/6 parental mice (i.e. *Nkr-p1a*, *Nkr-p1f*, *Ly-49c*, *CD69* and *Nkg2*) were identified in both hybrid strains. *Nkr-p1b* and *Nkr-p1c^{BALB}* gene isoforms of Balb/c were present in b+d-NK1.1neg mice, whereas *Nkr-p1d* and *Nkr-p1c^{B6}* of C57BL/6 origin in b-d+NK1.1+high

Table II. NKC domain genotype in novel mouse strains compared to parental C57BL/6 and BALB/c mice.

	Nkrp1 gene isoform						Ly49 gene isoform						
Strain	A	B^{Balb}	B^{B6}	C^{Balb}	C^{B6}	F	C	D	F	H	L	NKG2D	CD69
C57Bl/6 H2-Db+d-NK1.1high	+	-	+	-	+	+	+	+	+	+	-	+	+
C57Bl/6xBalb/c H2-Db+d-NK1.1neg	+	+	-	+	-	+	+	-	-	-	+	+	+
Balb/cxC57Bl/6 H2-Db-d+NK1.1high	+	-	+	-	+	+	+	+	+	+	-	+	+
Balb/c H2-Db-d+NK1.1neg	+	+	-	+	-	+	+	-	-	-	+	+	+

Presence or absence of individual *Nkrp-1* and *Ly-49* gene isoforms as well as *Nkg2d* and *CD69* genes are indicated with + or -, respectively.

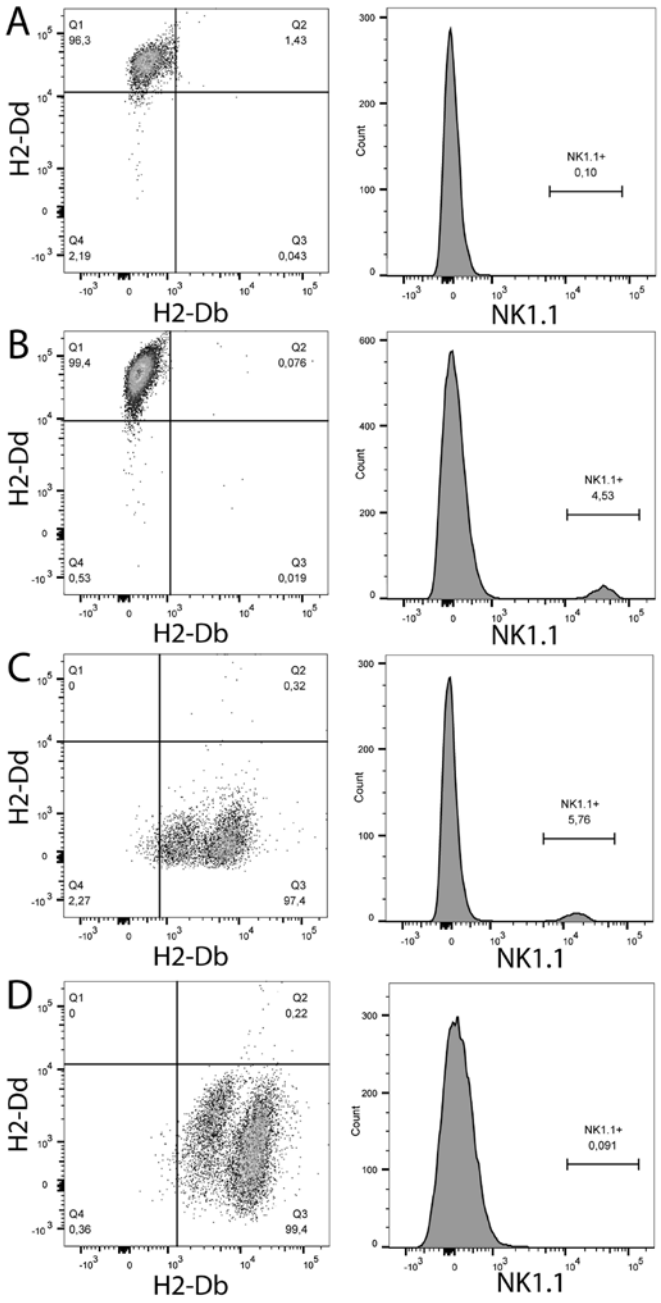

Figure 1. Phenotype of mouse strains. Blood cells with lymphocyte morphology gated on the basis of FSC/SSC were analyzed for NK1.1 and H2-D expression in (A) BALB/c, (B) H2-Db-d+/NK1.1 high, (C) C57BL/6 and (D) H2-Db+d-/NK1.1neg mice.

ones. These results together with the distribution of *Ly-49* gene family (Table II) show that the NKC domain was inherited as a whole.

The continuous FACS analysis was used for selection of the desired H2D and NK1.1 phenotypes (Fig. 1), which confirmed that mice of the novel strain with NKC^{B6} were NK1.1 high as they expressed NKR-P1C^{B6} receptor recognized by PK136 antibody (Fig. 1, histograms). The H2D haplotype was determined by anti-H2-Db and anti-H2-Dd specific antibodies (Fig. 1, dotplots).

The immune parameters of newly generated mouse strains. To reveal whether the novel strains differ from each other, as well as from parental strains in the proportion of basic immune cell subpopulations and their activation state, we evaluated the phenotype of blood cells using flow cytometry. We found significant differences in the percentage of NK, cytotoxic and helper T as well as B cells between the novel strains in comparison to parental mice. The NK cell proportion was significantly lower in b-d+NK1.1high vs Balb/c, but without changes between b+d-NK1.1neg and C57BL/6 strains (Fig. 2A). Evaluating the activation antigens (CD69 and NKG2D) on NK cells, the b-d+NK1.1high mice showed the highest (similar to that of Balb/c), whereas b+d-NK1.1neg the lowest proportion of $CD69^+$ NK cells (Fig. 2B), while the NKG2D was lower in both novel strains relative to parental ones (Fig. 2C). The NK cell-mediated cytotoxicity using spleen cells from tested mice as effectors against YAC-1 targets we found decreased NK cell lytic function in mice of the C57BL/6 H2-D background but not in mice of the Balb/c H2-D haplotype (Fig. 2D), which correlated with lower NKG2D expression. As for the distribution of cells of the adaptive immunity, the percentage of CTLs was higher in both novel strains compared to parental ones, while the CD4/CD8 ratio was the lowest in NKC^{B6}-bearing mice (Fig. 2E and F).

B cell number was comparable in the two novel mouse strains and was lower than that noted in C57BL/6 but higher than in Balb/c mice (Fig. 2G). Increased percentage of $CD86^+$ antigen presenting B cells was found in mice possessing the NKC^{BALB} domain, compared to those with NKC^{B6} (Fig. 2H).

Taken together, the most important immune changes in the novel strains lie in upregulation of CTLs, lower proportion of NK cells in NKC^{B6} mice, and the lowest expression of $CD69^+$ NK cells in b+d-NK1.1high strain.

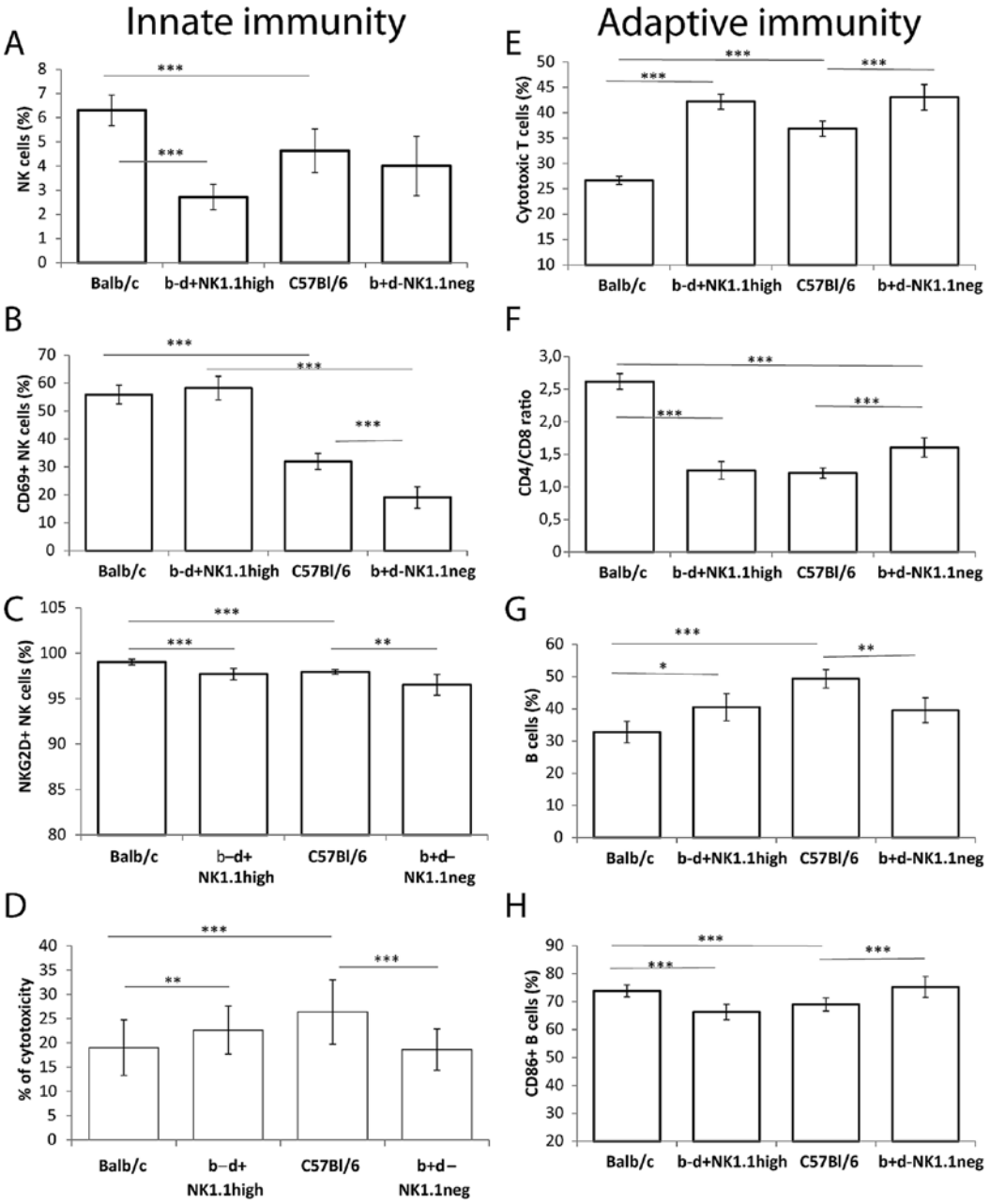

Figure 2. Distribution of basic immune cell subpopulations in the peripheral blood and NK cell activity. Phenotype and function. FACS analysis of NK cells, $CD3^-Nkp46^+$ (A), $CD69^+$ NK cells (B), and $NKG2D^+$ NK cells (C), distribution and the NK cell-mediated cytotoxicity against NK-sensitive YAC-1 tumor targets (D). Proportion of T cells $CD4^-CD8^+$ out of $CD3^+$ cells (E) and expressed as CD4/CD8 ratio (F) and proportion of B cells, $B220^+$ (G) and antigen-presenting activated B cells. $B220^+/CD86^+$ (H). Data represent average ± SD of values from 3 individual experiments (6-10 animals per group). **P<0.01, ***P<0.001, significant changes between individual mouse strains.

Resistance of novel mouse strains to tumor development. After the basic genetic and immunological characterization of the new strains, we concentrated on their reactivity to implanted and chemically-induced tumors. For this purpose, we first followed the development of C57BL/6-syngeneic B16F10 melanoma including primary tumors and metastases, and the Balb/c-syngeneic MCB8 fibrosarcoma. We compared the growth of these tumors in the syngeneic vs. hybrid mice of the same H2D background. In C57BL/6 mice, the expected progressive growth of B16F10 melanoma was observed from day 8. In b+d-NK1.1neg mouse strain, the tumor appeared later and grew slower (Fig. 3A). Intravenous administration of B16F10 tumor cells resulted in the development of a lower number of metastatic foci in the lung in b+d-NK1.1neg compared to C57BL/6 strain (Fig. 3B) counted on day 28. Evaluation of MCB8 fibrosarcoma growth using three different transplant sizes ($1x10^5$, $5x10^5$ or $1x10^6$ per mouse) showed that the tumor volume grew more rapidly in Balb/c than in b-d+NK1.1high mice after 10-12 days post inoculation (Fig. 3C and D).

To avoid the syngeneicity of tumor transplants to any of the parental mice, we further followed the development of chemically-induced (AOM+DSS) colorectal cancer, growing similarly in both parental strains. We revealed that unlike H2Db-d+NK1.1high mice, the H2Db+d-NK1.1neg strain was highly resistant to the development of this experimental cancer model (Fig. 4).

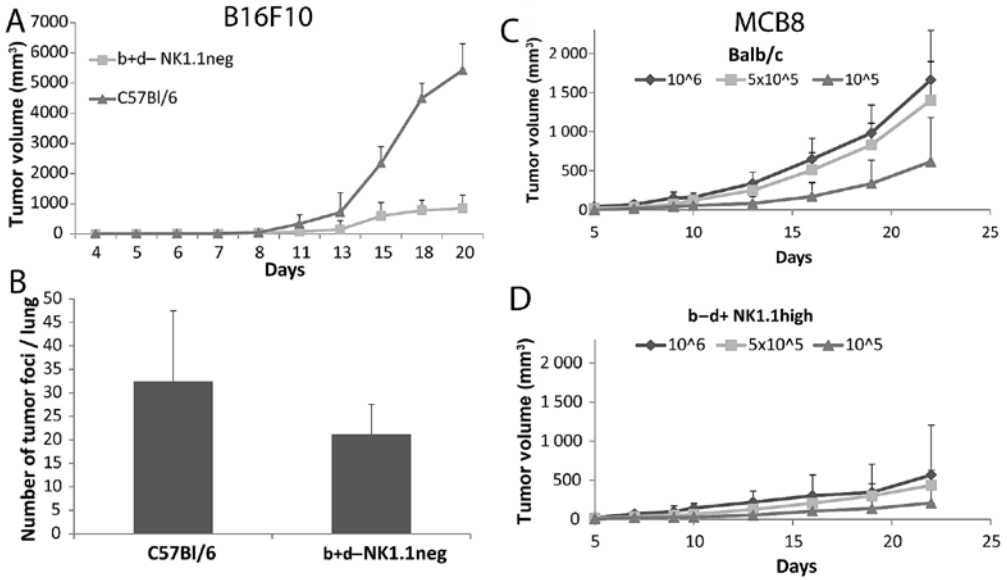

Figure 3. B16F10 melanoma and MCB8 fibrosarcoma growth. The B16F10 tumor growth (A) and number of metastatic foci per lung on day 28 (B) in C57BL/6 and b+d-NK1.1neg mice. The development of fibrosarcoma in Balb/c (C) and b-d+NK1.1high (D) mice after s.c. inoculation of $1x10^5$, $5x10^5$ or $1x10^6$ MCB8 fibrosarcoma cells. The tumor volume represents the average ± SD of values of three experiments performed (8-10 mice per group).

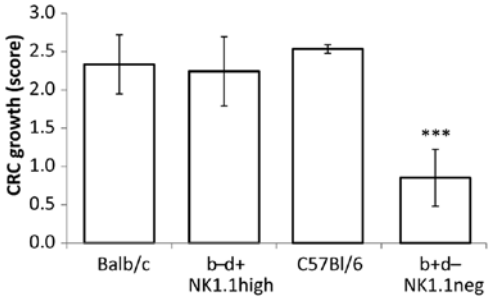

Figure 4. Development of chemically-induced colorectal cancer (CRC). The development of CRC was evaluated after 3 months using the following scoring of severity: 0, no tumors; 1, 1-5 tumors/colon; 2, 5-10 tumors/colon; 3, >10 tumors/colon. CRC growth (score) represents the average ± SD of 4 experiments performed (10-15 per group). ***P<0.001, significant changes between individual mouse strains.

Finally, we focused on the reactivity of the hybrid mice to H2-D-negative TC-1/A9 tumor (syngeneic to C57BL/6) mimicking HPV-16-associated neoplasms by expressing E6/E7 oncoproteins. As expected, TC-1/A9 tumor incidence was 100% in C57BL/6 mice, starting in the early stages, i.e. day 5 after tumor inoculation grew consistently (Fig. 5A and B). On day 15, when b+d-NK1.1neg mice were completely tumor-free as a consequence of rapid tumor regression observed from day 7 (Fig. 5C and D), the tumor incidence as well as the growth curve in b-d+NK1.1high peaked on day 10-12, then the tumors started shrinking (Fig. 5E and F). Moreover, the hybrid mice that rejected the primary tumors when rechallenged with the same dose of TC-1/A9 cells, did not develop any tumors whatsoever.

Significant differences in tumor incidence as well as the growth curve in the novel mouse strains were determined. While b+d-NK1.1neg mice started to develop and regressed the TC1/A9 tumor earlier, the b-d+NK1.1high mice exhibited, both later onset and postponed start of the regression phase. For better understanding of these features, we analyzed the accompanying immunological consequences.

Immunological changes in TC-1/A9 tumor-bearing animals. To detect the immunological changes ongoing in the parental C57BL/6 mice during TC-1/A9 tumor growth, and tumor regression in the novel mouse strains, we followed the distribution of basic immune cell subpopulations in blood and secondary lymphoid organs. The percentage of NK cells was significantly increased in the spleen and lymph nodes in both novel strains (to a greater extent in b+d-NK1.1neg), compared with C57BL/6 mice (Fig. 6A and B); they were not present in the blood (Fig. 6C). Significant rise of $NKG2D^+$ NK cells in tumor regressing novel strains was found in all tested immune compartments more pronounced in lymph nodes of b-d+NK1.1high mice (Fig. 6D-F). Furthermore, in TC-1/A9 tumor-bearing novel strains, the NK cell-mediated (against YAC-1 targets; Fig. 6G) as well as specific (against TC-1/A9 targets; Fig. 6H) cytotoxicity was lower on day 7, but linearly increased until day 18 compared with C57BL/6 mice. The cytotoxic activity was thus elevated during tumor regression. Analyzing the adaptive part of immunity the proportion of B cells increased only in the spleen (Fig. 7A), while the CD86 costimulatory molecule expression on B cells was raised in all examined organs comparing hybrid mice with the C57BL/6 strain (Fig. 7A, C and E). CD4/CD8 ratio in the spleen, lymph nodes, and blood was shifted towards helper T cells ($CD4^+$) in b+d-NK1.1neg strain (Fig. 7B, D and F), but not in the spleen b-d+NK1.1high, where the CTL ($CD8^+$) prevailed (Fig. 7B).

The increased number of both cytotoxic cells (NK and CTL) populations and expression of NKG2D activation antigen in the spleen correlated with upregulated lytic activity measured against NK-sensitive and specific TC1/A9 target cells. In addition to that, the enhancement of $CD4^+$ T helper, preferentially in b+d-NK1.1neg mice can participate in tumor rejection by cytokine synthesis as well as cytotoxic activity.

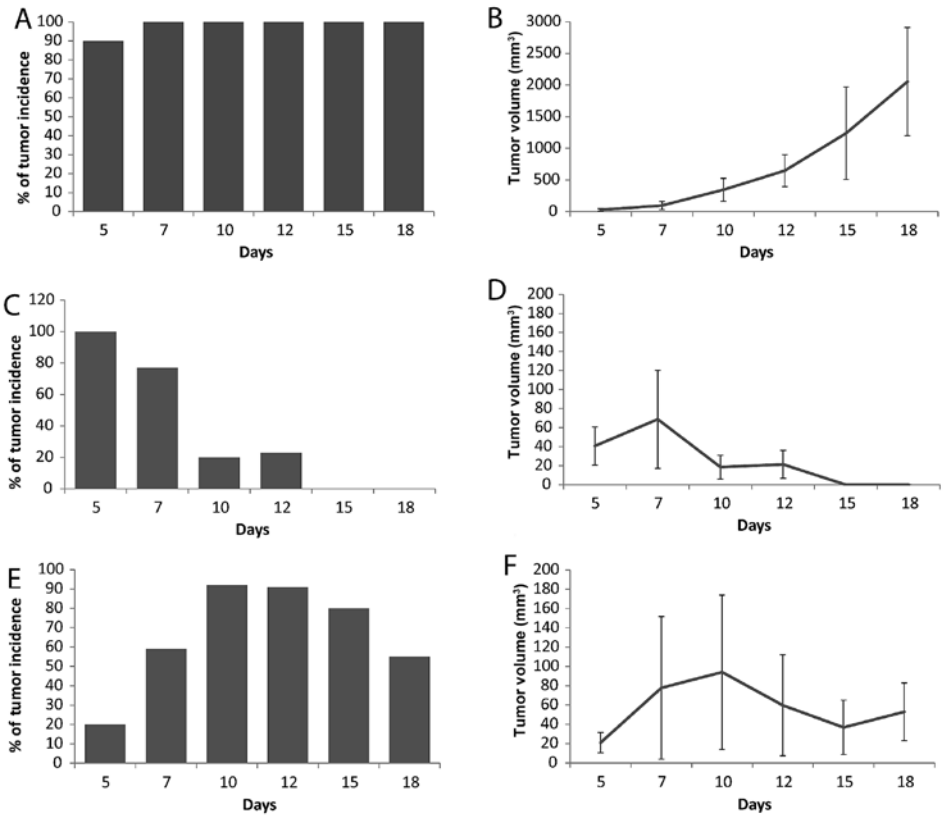

Figure 5. TC-1/A9 tumor incidence and growth. C57BL/6 (A and B), b+d-NK1.1neg (C and D) and b-d+NK1.1high (E and F) mice. The tumor incidence (percent of tumor-positive mice; A, C and E) and the tumor volume (B, D and F) depicted in graphs represent the average ± SD of values from individual mice (6-10 per group) of five experiments performed.

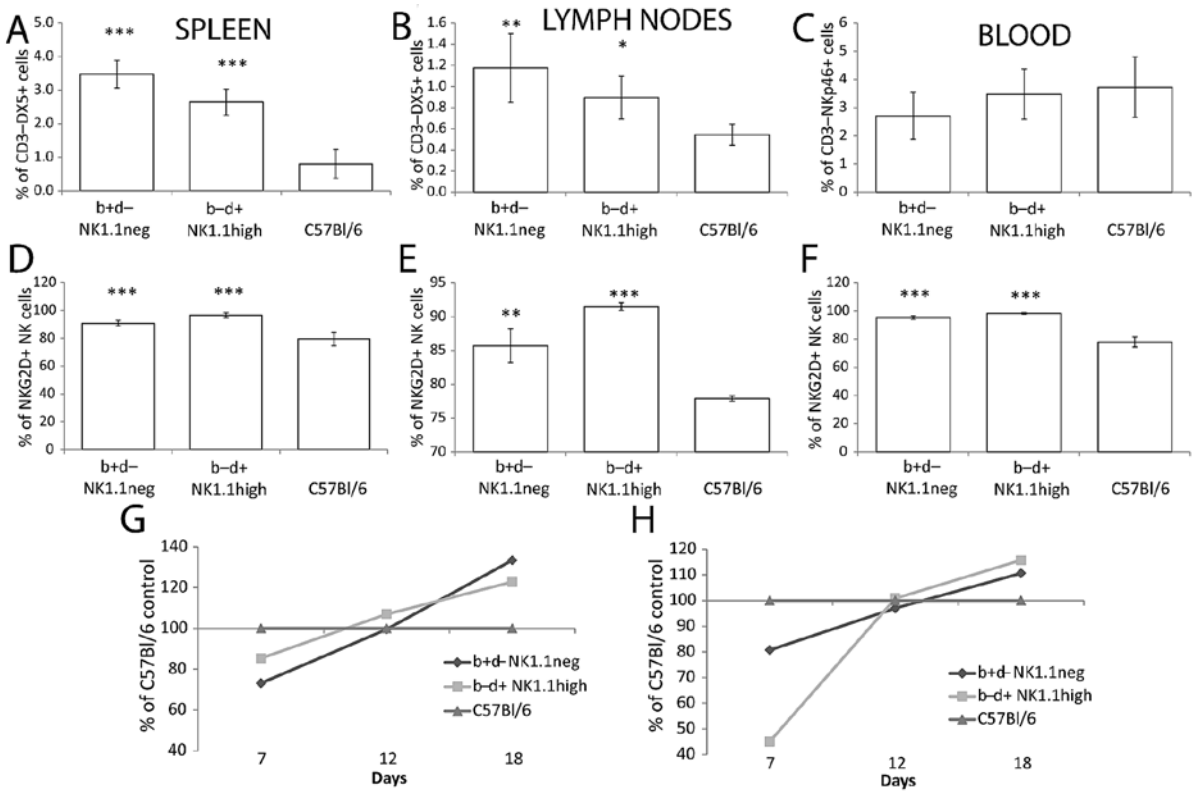

Figure 6. Immune changes during TC-1/A9 tumor development. The distribution of NK cells in the spleen, in lymph nodes ($CD3^-DX5^+$; A and B), in blood ($CD3^-Nkp46^+$; C), and $NKG2D^+$ NK cells (D, E and F) are shown. The spleen cell cytotoxicity was determined against NK cell-sensitive YAC-1 (G) and TC-1/A9 (H) tumor targets on the 7th, 12th and 18th day. The data are presented as percentage of control C57BL/6 mice stated as 100%, (triangles); b+d-NK1.1neg (squares); b-d+NK1.1high (diamonds). Data represent average ± SD of values from 5 individual experiments (6-10 animals per group). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, significant changes between novel and parental C57BL/6 mice.

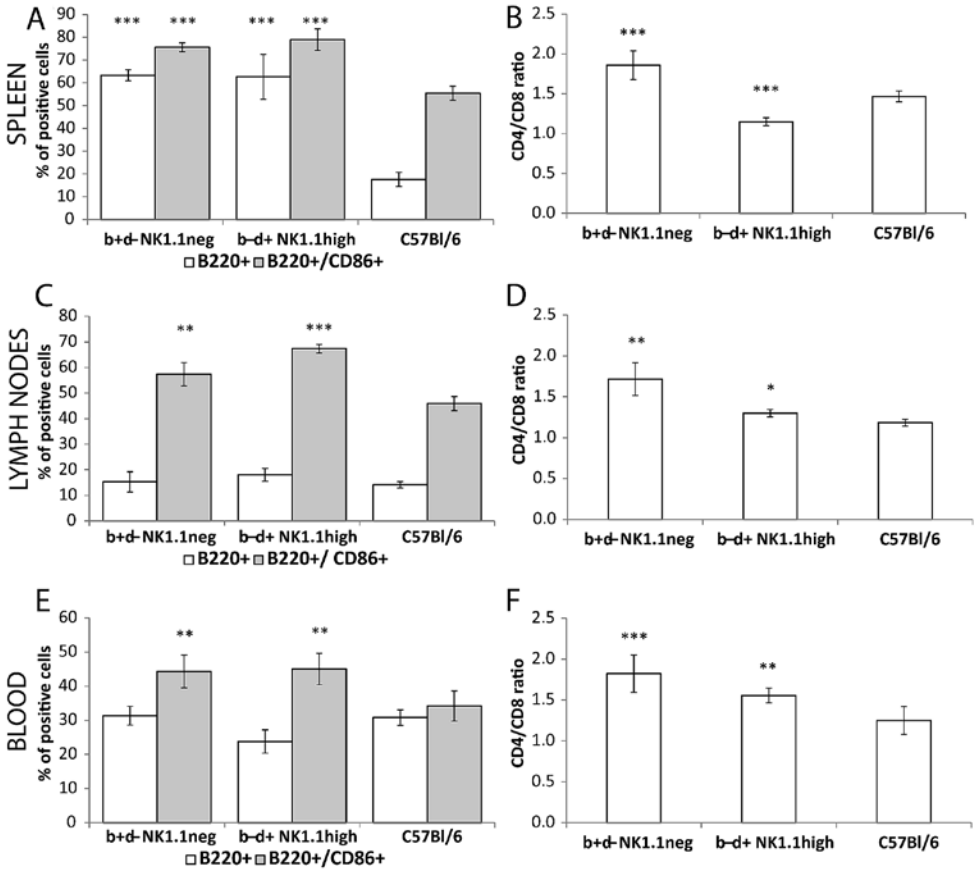

Figure 7. Distribution of B and T cell subpopulations of TC-1/A9 tumor-bearing mice. Percentages of B220$^+$ B cells (open columns), antigen-presenting B220$^+$/CD86$^+$ B cells (closed columns) (A, C and E) and T helper and T cytotoxic cells expressed as CD4/CD8 ratio (B, D and F). Data represent average ± SD of values from 3 individual experiments (10 animals per group). *P<0.05, **P<0.01, ***P<0.001, significant changes between novel and parental C57BL/6 mouse strains.

Discussion

In contrast to the adaptive immune system, receptors of the innate immune system are encoded in the germ-line. Extreme genetic polymorphism is manifested in the NKC locus encoding NK cell receptors (7,8). While such genetic diversity is frequently studied in infectious disease models (e.g. described resistance to MCMV or susceptibility to hypertension in congenic BALB.B6 mice with NKC^{B6}), the impact on cancer is mostly unknown (28,31,32).

The present study was designed to develop new strains of mice intercrossed between Balb/c and C57BL/6 parents distinct in the NKC domain, and the H2-D haplotype and to examine their sensitivity and immune response to transplanted (syngeneic to the parental mouse strains), as well as chemically-induced tumors.

We first focused on the genetic and immune characteristics of the newly generated mouse strains related to the NKC domain and H2-D haplotype. The results of DNA analysis suggest that the NKC domain is inherited as a whole, which corresponds with the proximity of *Nkrp1* and *Ly49* gene families in the NKC on chromosome 6 with the resulting strong linkage of the genes (33).

Phenotyping of healthy mice was concentrated on the genetic differences in NK, T and B cell populations between the strains, their activation markers (NKG2D, CD69 and CD86) and cytotoxic effector function. The proportion of NK cells, which was the highest in Balb/c mice, was significantly decreased after introduction of NKC^{B6} in the b-d+NK1.1high strain. The comparison of CD69 expression on NK cells in parental and novel mouse strains exhibited the same proportion in Balb/c and b-d+NK1.1high mice (55%), whereas significantly lower percentage was found in the C57BL/6 (30%) that was further reduced in b+d-NK1.1neg mice (20%). CD69 negatively regulates the production of IFN-γ, which is known to support antitumor immune response, and also upregulates the release of anti-inflammatory TGF-β1 by stimulated C57BL/6 CD4 T cells. We believe that the down-modulation of CD69 expression in b+d-NK1.1neg mice, similarly as anti-CD69 antibody treatment, could contribute to an increased antitumor response, and the resulting tumor regression (34).

The most important activation marker of NK cells, NKG2D (recognizing stress proteins on tumor cells) plays a key role in cancer recognition, immune surveillance and suppression of tumor progression (35-37). Comparing the novel and parental strains, we detected lower proportion of NKG2D$^+$ NK cells in the peripheral blood of healthy mice bearing NKC^{B6}, while the NK cell-mediated cytotoxicity was higher. The strain-specific differences could be influenced also by NKG2D ligands, constitutively expressed by many *in vitro*-adapted tumor cell lines and carcinogen-induced tumors, indicating that the expression of these ligands is induced after tumorigenesis, which enhances the susceptibility to NKG2D-dependent

NK-cell cytotoxicity and also primes tumor-specific cytotoxic T-cell responses (38-40).

Analyzing the T cell subsets, we detected a highly significant increase in the proportion of $CD8^+$ cytotoxic lymphocytes in the novel strains relative to the H2-D-matching parents (Balb/c vs. b-d+NK1.1high and C57BL/6 vs. b+d-NK1.1neg). Above that, the b+d-NK1.1neg had higher proportion of $CD4^+$ T cells playing an important role in tumor regression. The mechanism underlining the role of Th1 subsets lies in their capacity to activate macrophages, NK and $CD8^+$ T cells via IFN-γ production; the detected increase in $CD4^+$ T cell proportion in b+d-NK1.1neg mice thus corresponds with their resistance to tumor development (41).

The loss or downregulation of MHC class I molecules on tumor cells is a common mechanism by which tumors can escape T-cell mediated immune response, however, it makes these cells more susceptible to the NK cell-driven surveillance (4). We chose MHC class I-deficient tumor cell lines (tested by flow cytometry before the inoculation of mice), B16F10 melanoma and MCB8 fibrosarcoma derived from C57BL/6 and Balb/c mice, respectively, to follow the complex antitumor immune response and the engagement of the distinct sets of receptors encoded in the NKC domain in novel vs. parental strains. The B16F10 melanoma growth was significantly impaired, and lower number of tumor foci was detected in the lung using metastatic model of melanoma in the b+d-NK1.1neg strain compared to C57BL/6. This could be attributed to the upregulation of NKG2D in the novel strains and activation of cytotoxic effector cells that restrain the tumor growth (42,43). Similar effects were observed with MCB8 fibrosarcoma syngeneic to Balb/c. The tumor growth was much slower in b-d+NK1.1high mice bearing Balb/c H2D haplotype and NKC^{B6}, however, compared to the development of the above mentioned syngeneic tumor models, no regression was observed in the course of the period that followed.

Different situation occurred in colorectal cancer model induced by azoxymethane and DSS (29). Chronic inflammation can generate immunosuppressive microenvironment that is advantageous for tumor formation and progression characterized by accumulation of pro-inflammatory mediators, infiltration of immune suppressor cells, and activation of immune checkpoint pathways in effector T cells (44). Significantly impaired development of chemically-induced CRC was observed only in b+d-NK1.1neg mice that inherited the higher proportion of CD4 cells and the NKC^{BALB} domain. However, they also have lower number of $CD69^+$ NK cells, higher expression of NKG2D on NK cells, decreased percentage of $CD4^+CD25^+$ Tregs and $CD11b^+Gr1^+$ myeloid suppressor cells (data not shown) that could all contribute to the suppression of tumor growth.

For further immunological analyses, we used the moderately immunogenic TC-1/A9 cell line derived from H2-Db^+ TC-1 cells expressing E6/E7 oncoproteins of human papilloma virus 16 (HPV16) (42). Previously, we described the participation of activated NKT cells in the control of tumor growth at the early stages (45). The results of prophylactic immunization showed the distinct role of cell subsets in the development of immunity and the important role of NK cells (43).

Immune response to TC-1/A9 tumor cells demonstrated different dynamics of tumor initiation, peak and elimination processes in the two novel strains. In the b-d+NK1.1high mice the tumor development showed a postponed onset with maximum growth between day 10-12; after that, slow regression began, and on day 20 all animals were tumor free. In mice with C57BL/6 H2D haplotype the TC1/A9 tumor growth had a more rapid onset (90 to 100% of incidence on day 5), however, in the b+d-NK1.1neg mice the tumor started to regress rapidly on day 7, and on day 15 the animals were completely tumor-free, compared to parental C57BL/6, where the tumor grew continuously. Importantly, this tumor regression in the novel strains coincided with increased relative distribution of NK cells (2-3-times higher) in the spleens and lymph nodes, increased proportion of NKG2D-positive cells, as well as with upregulated spleen cell cytolytic activity against NK-sensitive YAC-1 cells, compared to the parental strain, suggesting the principle role of NK cells in the tumor rejection. We can also speculate that the low expression of CD69, upregulated NKG2D, and the higher CD4/CD8 ratio in b+d-NK1.1neg mice could be beneficial in the antitumor immune response. Under the TC-1/A9 tumor pressure we detected a 3-fold increase of B cells in the spleens of both novel mouse strains together with the higher percentage of $CD86^+$ B lymphocyte subpopulations compared to the parental C57BL/6. Activated $CD86^+$ B cells in tumor-bearing animals were also present in the blood and lymph nodes. These results account for the B cells proliferation and involvement of $CD86^+$ costimulatory molecule interacting with CD28 on T cells in communication/antigen presentation (46), supported also by increased proportion of $CD4^+$ T lymphocytes in all tested immune compartments.

Taken together we present here novel mouse strains different in the H2D haplotype and NKC domain and their new features exhibiting significant resistance to transplanted H2-Db-d-tumors. The b+d-NK1.1neg strain also showed resistance to chemically-induced colorectal cancer and its noteworthy association with selected immunological markers. Our results can thus serve as a basis for further studies of the tumor resistance mechanisms.

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