and TCR-β (H57-597) antibodies were obtained from eBioscience. The BD Bioscience Cytofix/Cytoperm kit was used for intracellular staining. Recombinant IL-12p70 and IL-15 and IL-15/IL15Rα were purchased from eBioscience and recombinant IL-18 was obtained from R&D systems.

***In vitro* stimulation assays**

NK cells were enriched using the NK cell isolation kit II mouse from Miltenyi Biotec and then purified using a FACS Aria II (BD Biosciences) and cultured with the indicated concentrations of cytokines or 10 ng/ml of PMA together with 1 μg/ml of ionomycin. After 16 h, cells were stained for flow cytometry analysis and/or supernatants were collected. Measurements of cytokine/chemokine production were performed using a CBA kit (BD Biosciences). Alternatively, NK cells were cultured for 4-5 days together with 10 ng/ml of IL-15/IL-15Rα (eBioscience). Then, NK cells were incubated in plates coated with anti-NK1.1 (clone PK136) or anti-Ly49D (clone 4E5) together with anti-CD107a antibodies and Golgi Plug and Golgi Stop (BD Bioscience). After 6 h, NK cells were stained for flow cytometry analysis.

In vitro cytotoxicity assay

Target cells were labeled with 100 μCi of ^{51}Cr (perkin Elmer) and co-cultured with IL15 stimulated NK cells at defined effector to target ratios. After 4 h incubation at 37°C, supernatant were harvested and the levels of ^{51}Cr were quantified by gamma counter (Wallac Wizard). The percentage of specific killing was determined using the following formula: (Sample ^{51}Cr release – spontaneous ^{51}Cr release) / (Maximum ^{51}Cr release – Spontaneous ^{51}Cr release) x 100.

Organ processing

Lungs and livers were perfused with PBS to remove peripheral blood and incubated with 1 mg/ml collagenase type IV (Sigma-Aldrich), 0.1 mg/ml hyaluronidase (Sigma-Aldrich) and 0.02 mg/ml DNase I (Roche) for 45 min at 37°C. Cell suspensions obtained from spleen, BM, lungs and livers were treated with ACK to remove erythrocytes.

BM chimera

WT or TLR3$^{-/-}$ mice received 2 irradiation doses of 552 cGy before being injected iv with 10 x 10^6 total BM cells from WT or TLR3$^{-/-}$ mice. Mice were treated with neomycin for 2 weeks and used for experiments 8 weeks after reconstitution.

Antibiotic treatment

WT or TLR3$^{-/-}$ mice were treated with Ampicillin (1g/L), Streptomycin (1g/L), Metronidazol (0.5g/L) and Vancomycin (1g/L) ad libidum for 3-4 weeks via the drinking water. Water containing antibiotic was exchanged every three days.

Tumor models

For the MCA-induced fibrosarcoma model, mice were injected sc with 100 µl of corn oil containing 100 µg of MCA (Sigma-Aldrich) on the right hind flank. Development of fibrosarcoma was monitored weekly. For primary tumor growth 1 x 10^5 B16F10, 1 x 10^6 MC38, 1 x 10^6 RMAS-MSV or 1 x 10^6 RMAS-Rae1β cells were resuspended in 100 µl of PBS and injected in sc on the right hind flank whereas 2 x 10^5 E0771 cells were injected into the 4th mammary fatpad. Tumor measurement was recorded as the product of two perpendicular diameters (mm^2). For experimental metastasis, mice were injected iv with 2 x 10^5 B16F10 resupended in 200 µl of PBS and after 14 days lung metastases were counted using a dissection microscope. For some experiments, mice were treated with anti-AsialoGM1 (Wako Chemicals), anti-IFN-γ (H22 from Robert Schreiber, Washington School

of Medicine, St Louis, MO, USA) or IgG control antibodies (100-250 μg ip) on the day prior to tumor injection, the day of tumor inoculation, and then once a week.

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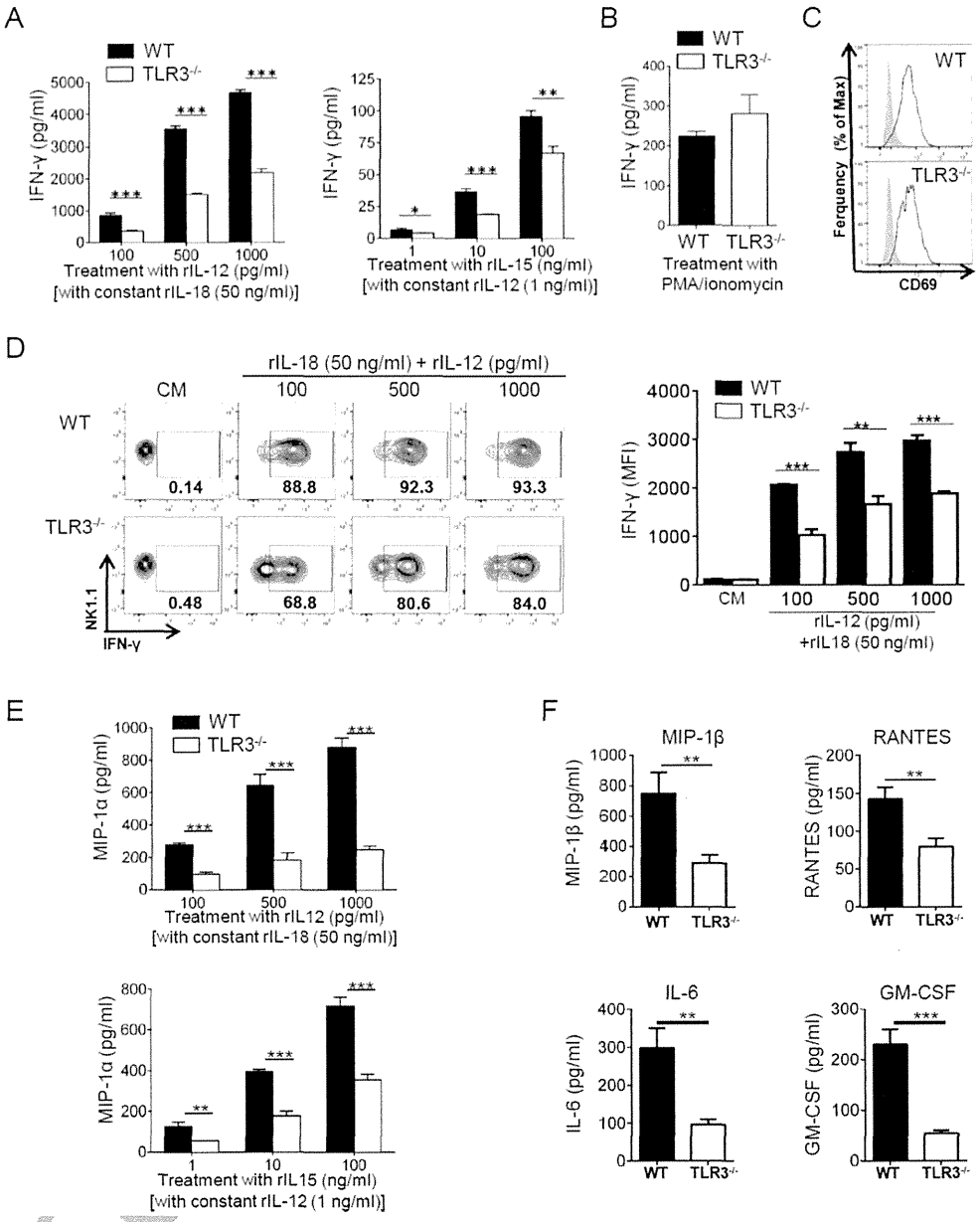

Figure 1: NK cells from TLR3$^{-/-}$ mice display defective cytokine production. NK cells were purified from the spleens of WT or TLR3$^{-/-}$ mice and cultured together with variable concentration of recombinant (r) IL-12 with constant rIL-18 (50 ng/ml) or with variable concentration of rIL-15 with constant concentration of rIL/12 (1 ng/ml) (A, D, and E), with 500 pg/ml of rIL-12 combined with 50 pg/ml of rIL-18 (C and F) or with 10 ng/ml of PMA

together with 1 μg/ml of ionomycin (B). After 16 h, supernatants were collected and IFN-γ (A, B), MIP-1α (E) or MIP-β, RANTES, IL-6 and GM-CSF (F) production was assessed by CBA. Alternatively, cells were stained and NK cell activation (C) or IFN-γ production (D) were analyzed by flow cytometry. Data shown are representative of at least two independent experiments and presented as mean ± SD. Mann-Whitney test was used to compare differences between mice (**, $p<0.01$; ***, $P<0.001$).

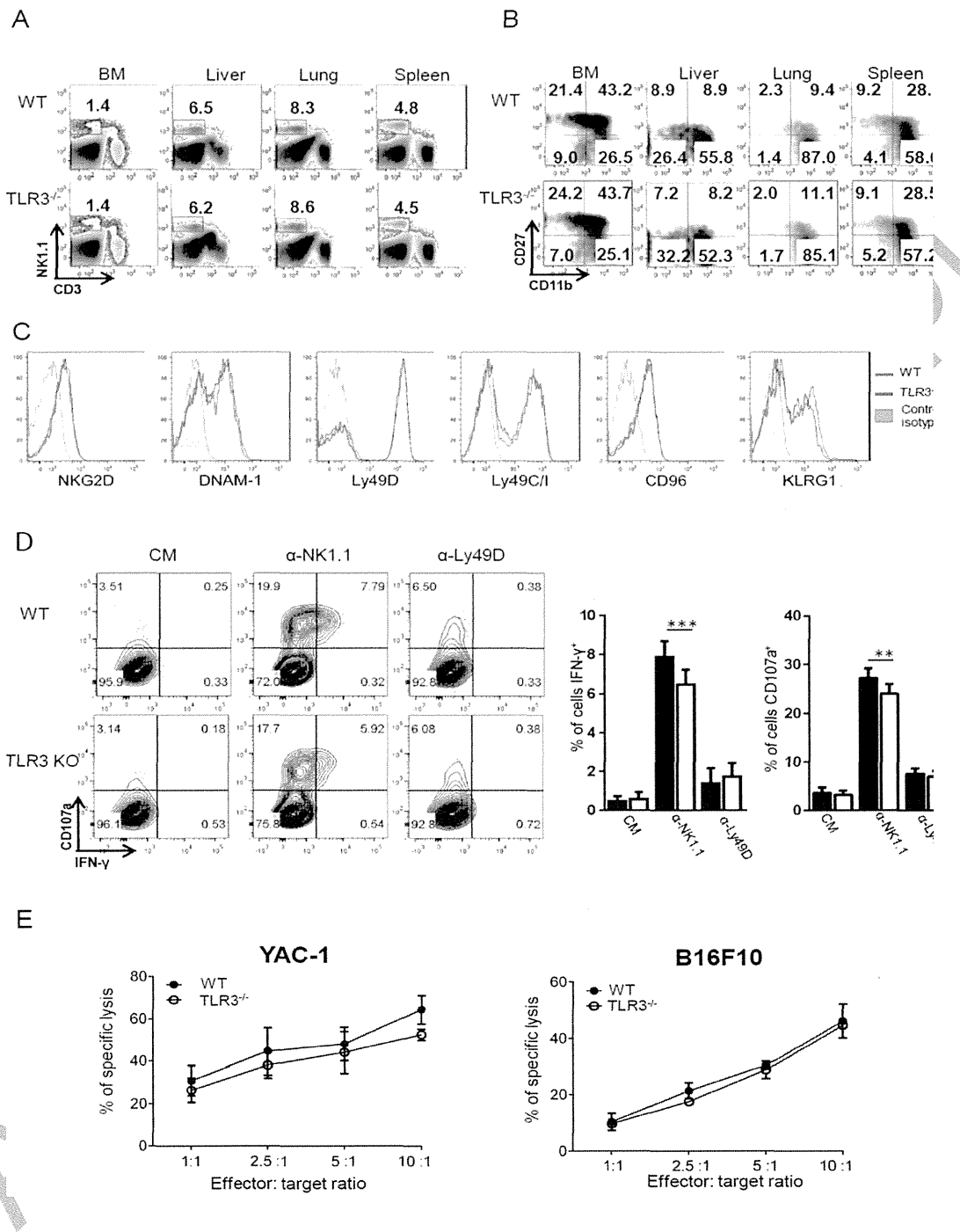

Figure 2: TLR3 is not required for NK cell development and acquisition of killing capacity. Bone marrow (BM), liver, lung and spleen from WT and TLR3$^{-/-}$ were processed and the percentages (A) and maturation status (B) of NK cells (CD3^{-}NK1.1^{+}) were

determined by flow cytometry. (C) Expression of activating and inhibitory receptors on splenic NK cells from WT (red) or TLR3$^{-/-}$ mice (blue). Data shown are representative of two independent experiments each consisting of three mice per group. (D and E) Purified splenic NK cells from WT or TLR3$^{-/-}$ mice were cultured for 3-5 days together with 10 ng/ml of IL-15/IL-15Rα. (D) NK cells were incubated in anti-NK1.1 or anti-Ly49D coated plates for 6 h and degranulation (measured by CD107a exposure) and IFN-γ production were assessed by flow cytometry. Representative dot plots are depicted and graphs represent pooled data from 2 (anti-Ly49D) to 3 (anti-NK1.1) experiments done in 3 to 5 experimental replicates. (E) Cytotoxic activity against YAC-1 or B16F10 cells was determined in a standard 4 h ^{51}Cr release assay. Graphs represent mean ± SD of triplicates from one experiment representative of 3 independent experiments.

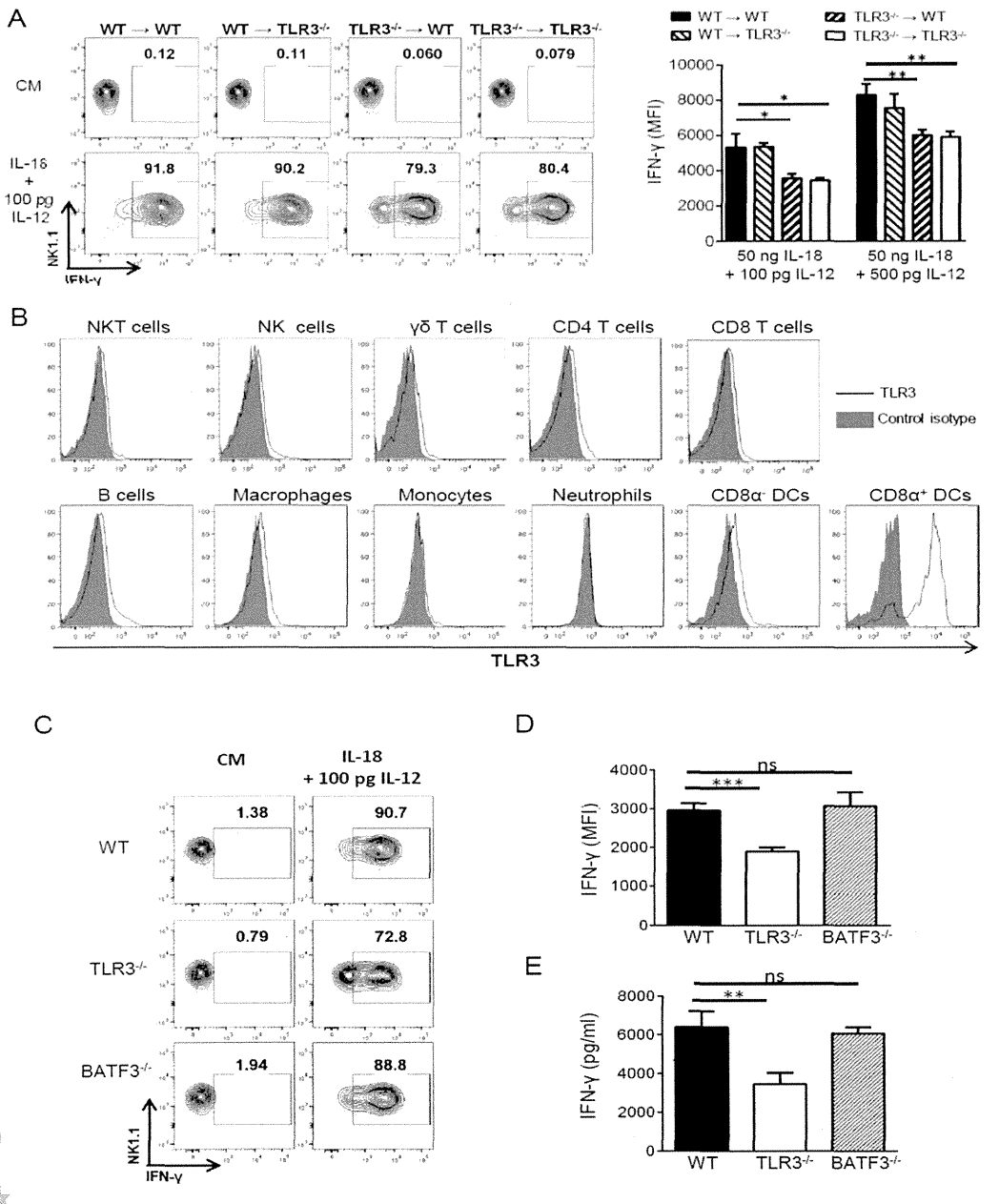

Figure 3: TLR3 expression on immune cells other than CD8α DCs is required for the acquisition of full NK cell functions. (A) Lethally irradiated WT mice were reconstituted with BM cells from WT or TLR3$^{-/-}$ mice. Alternatively, lethally irradiated TLR3$^{-/-}$ mice

received BM cells from WT or TLR3$^{-/-}$ mice. Eight weeks later, NK cells were isolated from the spleen, cultured for 16 h together with 50 ng/ml of rIL-18 and variable concentrations of rIL-12. IFN-γ production was determined by flow cytometry. Graphs and dot plots from one representative experiment out of 2 are depicted. (B) TLR3 expression by splenic NKT cells (TCRβ^{+} CD1d/α-GalCer tetramer^{+}), NK cells (CD3^{-} NK1.1^{+}), γδ T cells (TCRγδ^{+} CD3^{+}), CD4 T cells (TCRβ^{+} CD4^{+}), CD8 T cells (TCRβ^{+} CD8^{+}), B cells (CD19^{+} B220^{+}), macrophages (CD11b^{+} F4/80^{+}), monocytes (CD11b^{+} Ly6C^{high}), neutrophils (CD11b^{+} Ly6G^{+}), CD8α^{-} DC (CD11c^{+} MHC-II^{+} CDα8^{-}) and CD8α^{+} (CD11c^{+} MHC-II^{+} CD8α^{+}) from WT mice was determined by intracellular staining. (C-E) NK cells were purified from the spleen of WT, TLR3$^{-/-}$ and BATF3$^{-/-}$ mice and cultured in the presence of 100 pg/ml of rIL-12 and 50 ng/ml of rIL-18 for 16 hours. IFN-γ production was determined by flow cytometry (C and D) and by CBA (E). Representative dot plots and graphs showing the mean ± SD of triplicates from one experiment representative of 3 independent experiments are depicted. Mann-Whitney test was used to compare differences between mice (*, $p<0.05$; **, $p<0.01$; ***, $P<0.001$).

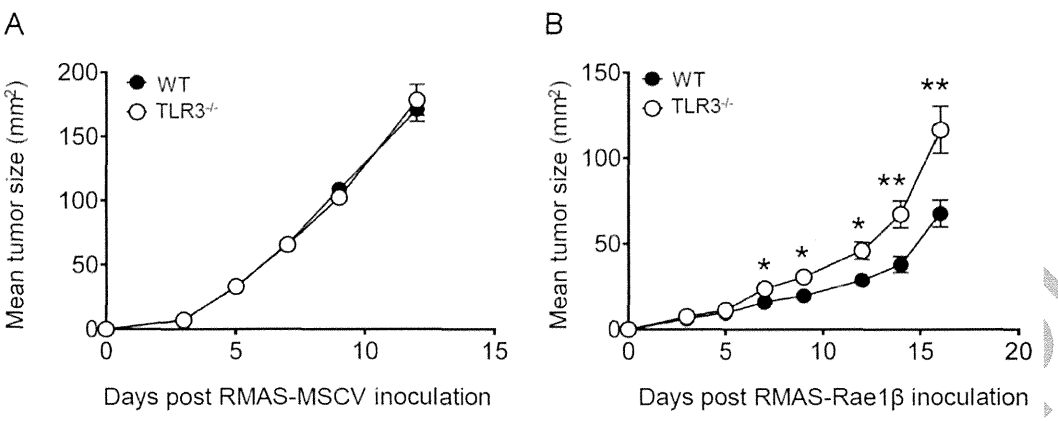

Figure 4: NKG2D-mediated suppression of tumor is attenuated in TLR3$^{-/-}$ mice. WT or TLR3$^{-/-}$ mice were injected sc with (A) RMAS-MSCV or (B) RMAS-Rae1β tumor cells and tumor size was recorded. Data shown are representative of 2 independent experiments with 5-10 mice per group and presented as mean ± SEM. Mann-Whitney test was used to compare differences between mice (** $p<0.01$; * $p<0.5$).

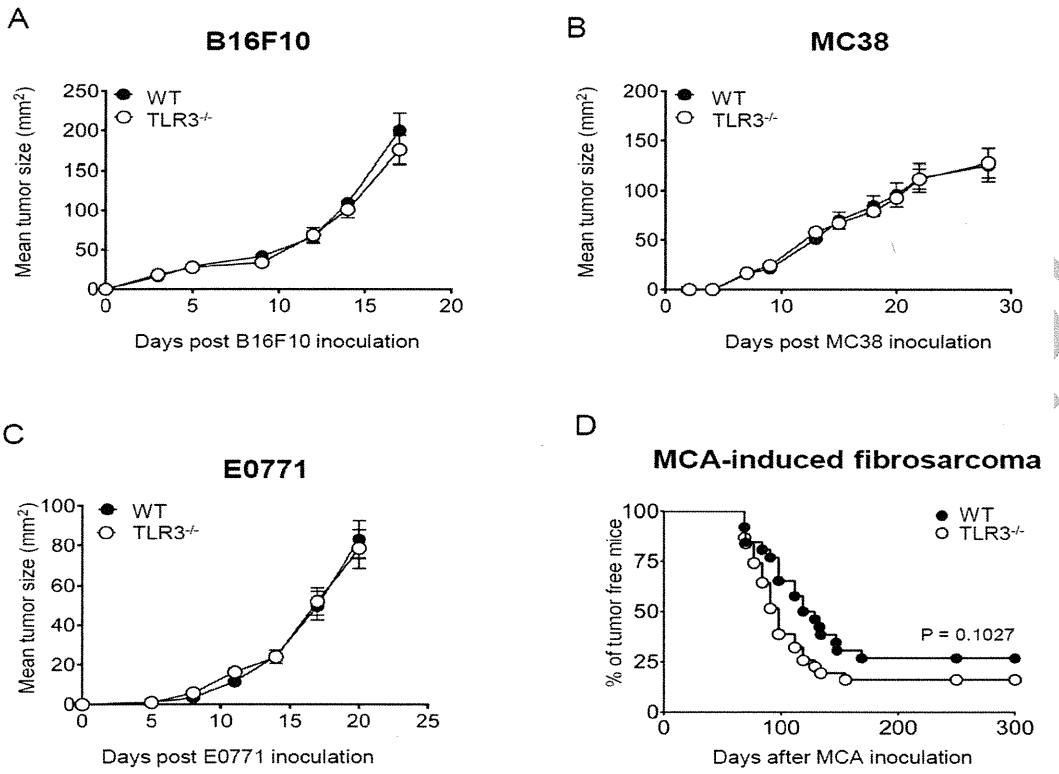

Figure 5: TLR3 is dispensable for the control of primary tumor growth and carcinogenesis. WT and TLR3$^{-/-}$ mice were injected sc with (A) B16F10 or (B) MC38 tumor cells (n=5 mice per group). (C) Mice were orthotopically injected into the mammary fat pad with E0771 cells (n=10 mice per group). Data shown are representative of 2 independent experiments and presented as mean ± SEM. (D) WT and TLR3$^{-/-}$ mice were injected with 100 µg of MCA and subsequently monitored for tumor development over 300 days. Results are pooled from 2 independent experiments with a total of 26 WT and 31 TLR3$^{-/-}$ mice. Statistical differences in tumor incidence were determined by Mantel Cox Log-rank test.

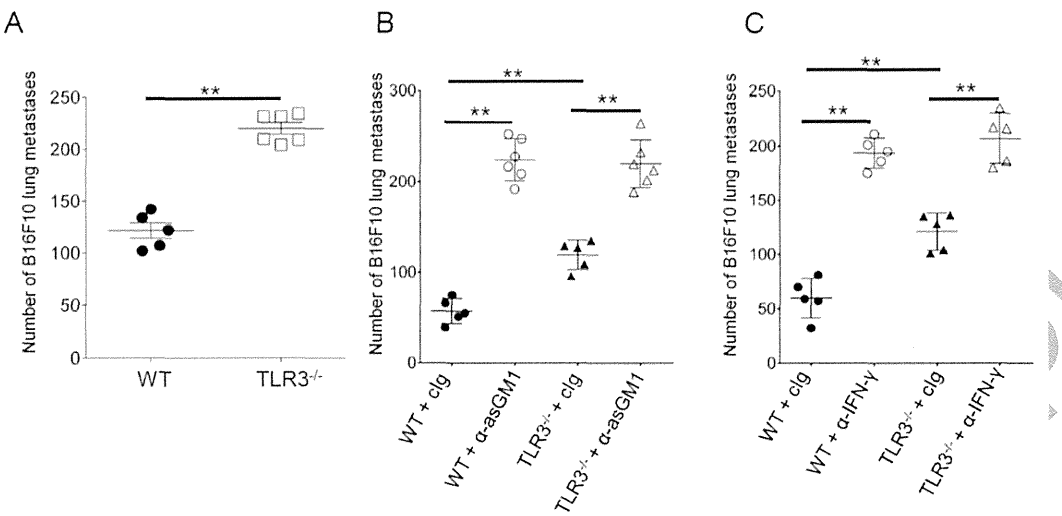

Figure 6: TLR3 participate to the control of metastasis in a NK cell- and IFN-γ-dependent manner. WT and TLR3$^{-/-}$ mice were challenged iv with B16F10 melanoma cells. For some experiments, mice were treated with NK cell-depleting anti-asialoGM1 antibodies (B) or blocking anti-IFN-γ (C). Symbols represent the number of lung metastases for individual mice 14 days following tumor inoculation. Mann-Whitney test was used to compare differences between mice (**, $P<0.01$).

Identification of a novel HLA-A*02:01-restricted cytotoxic T lymphocyte epitope derived from the EML4-ALK fusion gene

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Abstract. Cancer immunotherapy is a promising new approach to cancer treatment. It has been demonstrated that a high number of tumor-specific cytotoxic T cells (CTLs) is associated with increased disease-specific survival in lung cancer patients. Identification of superior CTL epitopes from tumor antigens is essential for the development of immunotherapy for malignant tumors. The EML4-ALK fusion gene was recently identified in a subset of non-small cell lung cancers (NSCLCs). In this study we searched for HLA-A*02:01- and HLA-A*24:02-restricted epitopes derived from EML4-ALK by screening predicted EML4-ALK-derived candidate peptides for the induction of tumor-reactive CTLs. Nine EML4-ALK-derived peptides were selected by a computer algorithm based on a permissive HLA-A*02:01 or HLA-A*24:02 binding motif. One of the nine peptides induced peptide-specific CTLs from human peripheral blood mononuclear cells. We were able to generate a peptide-specific CTL clone. This CTL clone specifically recognized peptide-pulsed T2 cells and H2228 cells expressing HLA-A*02:01 and EML4-ALK that had been treated with IFN-γ 48 h prior to examination. CTL activity was inhibited by an anti-HLA-class I monoclonal antibody (W6/32), consistent with a class I-restricted mechanism of cytotoxicity. These results suggest that this peptide (RLSALESRV) is a novel HLA-A*02:01-restricted CTL epitope and that it may be a new target for antigen-specific immunotherapy against EML4-ALK-positive cancers.

Introduction

Lung cancer is one of the main causes of cancer-related mortality. Approximately 85% of lung cancers are diagnosed as non-small cell lung cancer (NSCLC), and the overall survival (OS) rate for advanced NSCLC is poor. The 5-year survival rate is 5% for stage IIIb NSCLC and <1% for stage IV NSCLC (1). Treatment for NCSLC is determined by the patient's clinical and tumor characteristics, performance status (PS), the histological subtype and tumor genotype/phenotype.

Recently, there have been many studies concerning agents that target molecular changes, such as mutations in the epidermal growth factor receptor (EGFR) and the fusion oncogene EML4-ALK, in which the echinoderm microtubule-associated protein-like 4 (EML4) is fused with the intracellular domain of anaplastic kinase (ALK) (2-4). Although significant advances have been made in the treatment of NSCLC using molecular targeted therapies such as erlotinib and crizotinib, the median OS for patients with advanced NSCLC remains low (5,6), and acquired resistance to target agents is a major clinical problem. Therefore, the development of novel therapies is needed (7).

Immunotherapy manipulates the immune system to control and eradicate cancer. Many recent studies provide evidence suggesting that immunotherapeutic manipulations are viable in many tumor types, including lung cancer. Numerous trials of peptide vaccines, autologous cellular therapy, T cell-directed antibody therapy and monoclonal antibody therapy for lung cancer have been carried out around the world (8-10) and some of them have shown favorable results (11-13).

The EML4-ALK fusion gene was identified in NSCLC patients by a team led by Professor H. Mano. This fusion gene was formed as the result of a small inversion within the short arm of chromosome 2 that joins differing portions of the EML4 gene with a portion of the ALK gene (14,15). As a result of this fusion, constant dimerization of the kinase domain of ALK is induced and its catalytic activity increases consequently.

The EML4-ALK fusion gene is mainly identified in young, never/former light smokers with NSCLC (16). It is estimated that approximately 5% of all NSCLC cases have this fusion gene. A few reports have also identified EML4-ALK in other cancers, namely breast cancer and colorectal cancer (17,18). For the most part, the EML4-ALK fusion gene and other mutations, such as those in EGFR and KRAS, are mutually exclusive (19).

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Key words: EML4-ALK, peptide vaccine, CTL clone, lung cancer