Low-dose aspirin delays an inflammatory tumor progression in vivo in a transgenic mouse model of neuroblastoma

Lena-Maria Carlson1,2,*, Agnes Rasmuson1, Helena Idborg2, Lova Segerström1, Per-Johan Jakobsson3 Baldur Sveinbjörnsson1,2 and Per Kogner1

1Department of Women’s and Children’s Health, Karolinska Institutet, Stockholm, S-17176, Sweden. 2Department of Oncology-Pathology, Karolinska Institutet, Stockholm, S-17176, Sweden. 3Department of Medicine, Karolinska Institutet, Stockholm, S-17176, Sweden. *To whom correspondence should be addressed. Tel: +46 8 517 73534 (office); Fax: +46 8 517 73475; Email: Lena-Maria.Carlson@ki.se

Tumor-associated inflammation is a driving force in several adult cancers and intake of low-dose aspirin has proven to reduce cancer incidence. Little is known about tumor-associated inflammation in pediatric neoplasms and no in vivo data exists on the effectiveness of low-dose aspirin on established tumors. The present study employs the transgenic TH–MYCN mouse model for neuroblastoma (NB) to evaluate inflammatory patterns paralleling tumor growth in vivo and low-dose aspirin as a therapeutic option for high-risk NB. Spontaneously arising abdominal tumors were monitored for tumor-associated inflammation ex vivo at various stages of disease and homozygous mice received daily low-dose aspirin (10 mg/kg) using oral gavage or no treatment, from 4.5 to 6 weeks of age. Using flow cytometry, a transition from an adaptive immune response predominated by CD8+ T cell in early neoplastic lesions, towards enrichment in immature cells of the innate immune system, including myeloid-derived suppressor cells, dendritic cells and tumor-associated macrophages, was detected during tumor progression. An M1 to M2 transition of tumor-associated macrophages was demonstrated, paralleled by a deterioration of dendritic cell status. Treatment with low-dose aspirin to mice homozygous for the TH–MYCN transgene significantly reduced the tumor burden (P < 0.01), the presence of tumor-associated cells of the innate immune system (P < 0.01), as well as the intratumoral expression of transforming growth factor-β, thromboxane A2 (P < 0.05) and prostaglandin D2 (P < 0.01). In conclusion, tumor-associated inflammation appears as a potential therapeutic target in NB and low-dose aspirin reduces tumor burden in the TH–MYCN transgenic mouse model of NB, hence warranting further studies on aspirin in high-risk NB.

Introduction

It is now widely appreciated that the onset of cancer involves an intricate interplay between the malignantly transformed cells and the surrounding stroma, where the rules for survival and expansion are often modified and defined by the growing tumor. As a part of the stroma and the tumor microenvironment, cells from the immune system are known to represent major players in the given interplay, with an intrinsic ability to undermine cancer cells on their route to expansion. Yet, the immune system often fails this given task and is indeed subverted by the tumor to promote its expansion and progression (1,2). An inflammatory component has been suggested to represent “the seventh hallmark of cancer,” (3) and its importance in the pathogenesis of cancer has been demonstrated by a decreased risk for a number of adult cancers in long-term users of NSAIDs (4–5). Importantly, it was shown that lower doses (75–81 mg daily) of aspirin, a dual COX-1/COX-2 inhibitor, were as effective as higher, minimizing the risk of side effects (6–9). To our knowledge, no studies so far have evaluated low-dose aspirin on established tumors in vivo. Furthermore, less is known about the contribution of inflammation to tumorigenesis in pediatric cancers.

Neuroblastoma (NB) stands as the most common extracranial solid tumor of early childhood, with a median age of onset of 17 months. The incidence is 10.5 per million children <15 years of age per year, with little variation between Europe and North America, and there are 700 new cases diagnosed in the USA each year (10,11). Clinically, NB is known to display a diverse repertoire of behaviors, ranging from spontaneous regression to progressive, disseminated disease in high-risk patients in spite of intense multimodal therapy, with only 40% long-term survival rate in this group (12). Alternative approaches are being pursued for these patients, including immunotherapy employing the chimeric ch14.18 monoclonal anti-GD2 antibody in combination with IL-2 and GM-CSF, which is now being included into standard protocols (13).

In the context of antitumor immunity and tumor-promoting inflammation, the immune system has a dual role, given its intrinsic ability to defeat, and yet within the same tumor support and nourish the expanding mass of tumor cells. Myeloid cells such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) are known to inhibit antitumor immunity and promote tumor growth, whereas some lymphoid cells such as subsets of T and natural killer cells are capable of exerting direct antitumor immunity (1,14,15). In the case of NB, the immunological signature will be of utmost importance when dictating responses seen to treatment with ch14.18, yet few studies have scrutinized how NB tumors interact with their surrounding stroma. Furthermore, previous work from our group demonstrated that COX-2 inhibitors could delay NB tumor growth in vivo (16), suggesting that unfavorable inflammatory patterns may prevail and promote NB growth. Therefore, the liability of the immune system on site and the susceptibility of high-risk NB to the applied immunotherapy at different stages of disease remains unclear.

In this study, we employed the transgenic TH–MYCN mouse model, known for its resemblance to human NB and with targeted expression of MYCN in cells of the neural crest (17–19), to monitor tumor-associated inflammation during tumor progression and to evaluate low-dose aspirin as a potential new treatment option for high-risk NB patients. Our results highlight a progressive deterioration of a TH2 skewed tumor microenvironment with concomitant alterations in the composition of tumor-infiltrating hematopoietic cells in favor of immature cells of the innate immune system and towards a suppression of adaptive immunity. Furthermore, our study demonstrates the potential to employ anti-inflammatory treatment using low-dose aspirin as a novel auxiliary treatment modality for NB.

Materials and methods

Mice and treatment

The TH–MYCN animals were obtained from the Mouse Model of Human Cancer Consortium Repository as an N16 backcross to the 129X1/SvJ background and have been kept as a continuous inbreeding. All animal experiments were in accordance with the Animal Protection Law (SFs1988:534), the Animal Protection Regulation (SFs1988:539), the Regulation for the Swedish National Board for Laboratory Animals (SFs1988:541), and approved by the regional ethics committee for animal research (ethical permit N39/08 and N26/11). Homozygous mice were sacrificed at 5 or 6 weeks of age and heterozygous mice with tumors of varying size were included. Each mouse received abdominal palpations three times.
times weekly to follow tumor development and animals were monitored closely for signs of discomfort.

Homozygous mice were randomized at 4.5 weeks of age to receive either no treatment (control, n = 15) or daily low-dose aspirin (10 mg/kg, n = 8), by oral gavage for 10 consecutive days, and killed at the age of 6 weeks. One untreated control and one treated did not fulfill the study due to signs of discomfort and were omitted from subsequent analyses. At sacrifice, animals were euthanized in carbon dioxide.

Genotyping

Animals were biopsied and genotyped using a modified version of the PCR protocol previously published (20). DNA was extracted from ear clips and/or tail biopsies using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The PCR was performed in a total reaction volume of 20 μl containing 1x HotStarTaq® master mix (Qiagen), 0.2 μM of each primer (Supplementary Table 1, available at Carcinogenesis Online), 2% dimethyl sulfoxide (BD Biosciences) and 500 ng of DNA. PCR products were separated by 1.5% agarose gel electrophoresis and detected by UV light.

Chemicals, antibodies and reagents

Aspirin (Sigma, St Louis, MO) was dissolved in 99.5% ethanol and further diluted in sterile water, keeping the final concentration of ethanol <3%. For flow cytometry antibodies and TaqMan® primers, see Supplementary Table 1, available at Carcinogenesis Online.

Preparation of tumor tissue

Tumors were isolated and dissected, and adrenals and/or kidneys were removed. Tumor tissue was divided and subjected to flow cytometry staining, stored at −80°C for further analyses or fixed in 4% paraformaldehyde for immunohistochemical staining.

Flow cytometry

Tumor tissue was mechanically digested into single-cell suspension using cell strainers (BD Biosciences, San Jose, CA), subsequently washed and red blood cells were removed using red-blood-cell lysis buffer (BD Pharmlyse, BD Biosciences). 300,000 cells were incubated with antibodies in phosphate-buffered saline 0.1% BSA prior to washing in phosphate-buffered saline 0.1% BSA. For intracellular staining, cytofix/cytoperm (BD Biosciences) was used for fixation and permeabilization. Fluorescence was detected using an LSRII flow cytometer (BD Biosciences) and subsequent analyses were performed using FlowJo software (Treestar, Ashland, OR). Isotype controls were used for all stainings but gating strategies included dead cell discrimination using dead cell dye (Supplementary Figure 1B, available at Carcinogenesis Online).

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and graded alcohols, hydrated, and were incubated for 5 min in 0.5% H2O2 in phosphate-buffered saline. After antigen retrieval in sodium citrate buffer (pH 6.5) in a microwave oven, the endogenous peroxidase was blocked by 0.3% H2O2 for 10 min. Sections were incubated overnight at 4°C with the antibody Ki-67 (NeoMarkers, Fremont, CA), anti-human/mouse CD3 (DAKO, Glostrup, Denmark) or mouse monoclonal anti-COX-1 (Abcam, Cambridge, UK). Sections were subsequently washed and processed with anti-rabbit HRP-SuperPicture

Quantitative real-time reverse transcription–PCR analyses

RNA was prepared from tumors using magnetic digestion and RNAeasy mini-kit (Qiagen) and complementary DNA was synthesized from 1 μg of RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Boston, MA). The total reaction volume was 25 μl, containing 1xTaqMan® Universal PCR Master Mix, 1xTaqMan® Gene Expression Assay (Supplementary Table 1, available at Carcinogenesis Online; Applied Biosystems) and 2 μl complementary DNA for detection of hypoxanthine phosphoribosyltransferase (HPRT) or 10 μl complementary DNA for detection of cytokines, all performed in MicroAmp optical 96-well plates covered with MicroAmp optical caps (Applied Biosystems). The messenger RNA expression levels were detected on an ABI PRISM 7500 sequence detection systems (Applied Biosystems). Primer efficiency was calculated and corrected for if cd if two or three of three CT values were equal to or above 40, the level of expression was considered undetectable. For each sample, the ΔCT value between the relevant target gene and HPRT was calculated as ΔCT = (CTtarget – CTHPRT). To determine the relative expression in aspirin treated tumors versus controls, the 2^(-ΔΔCT) method was used. All quantitative RT–PCR experiments included a no-template control and were performed in triplicate.

Prostanoid profiling by liquid chromatography tandem mass spectrometry

Approximately 30 mg tumor tissue was used and deuterated standards of prostaglandin (PG) E2, PGE2, PF9, thromboxane (TX) B2 (a stable metabolite of TXA2) and 6-keto-PGF1a were added prior to liquid–liquid extraction. Liquid–liquid extraction was performed on tissue homogenate, that is the tissue was mixed in 20 μl methanol, 125 μl 0.1% formic acid in MiliQ water for approximately 5 min. An aliquot of 600 μl ethyl acetate was used for extraction and repeated twice. Prostanoid analysis was performed by liquid chromatography tandem mass spectrometry on a Waters 2795 HPLC coupled to an Acquity TQ Detector triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The mobile phase was composed of MiliQ water as solvent A and acetonitrile acidified with 0.05% formic acid as solvent B. Separation of the analytes was achieved on a Synergy Hydro-RP column (100 mm, 2 mm intradimensionally, 25 μm particle size and 100 Å pore size) by a 45 min linear stepwise gradient running from 10% to 90% B. Prostanoids were detected in multiple reaction monitoring mode recording mass transitions of mz 351.1 > 315.1 for PGE2 and for PGI2 eluting at 23.2 min and 24.2 min, respectively, mz 353.2 > 301.1 at 22.6 min for PGF2α, mz 369.1 > 169.1 at 21.6 min for TXB2 and mz 369.1 > 245.2 at 17.0 min for 6-keto-PGF1a. Internal standard calibration curve was used for calibration.

Statistics and preparation of graphs

All statistics and graphs were prepared using GraphPad prism, version 5 (GraphPad software Inc., San Diego, CA). All comparisons between two groups were performed with two-sided Mann–Whitney U-tests. Correlation was assessed using non-parametric two-sided Spearman test.

Results

TH-MYCN tumors are subjected to early infiltration by T cells with a preferential accumulation of CD4+ T-helper cells

The TH-MYCN tumors were reported previously to be non-immunogenic and absent of lymphocytic infiltration (22). Yet in our hands, CD3+ tumor-infiltrating T cell were detected by immunohistochemistry, as well as by flow cytometry, in tumor lesions of 5 and 6 week old homozygous mice (Figure 1A and B). However, when evaluating the infiltrating hematopoietic compartment (defined as dead cell stain/CX345, Supplementary Figure 1B, available at Carcinogenesis Online) in NB tumors of different stages a prominent diminution of the CD3+ compartment paralleled tumor progression. As depicted in Figure 1B, CD3+ T cells constituted a prominent proportion of all infiltrating cells at early stages of disease (median 63%), whereas the same population was outnumbered in advanced bulky NB tumors (median 27%) in favor of other subsets of infiltrating cells. In parallel, an increase in the CD4/CD8 ratio within the CD3+ compartment was observed during our monitored time window (Figure 1C), indicating a redistribution of the T cell compartment in the course of NB development, toward diminishment of CD8+ cytotoxic T-lymphocyte responses.

Using flow cytometry, we monitored immunosuppressive regulatory T cells (Tregs, defined as dead cell stain/CX347/Foxp3+) within TH-MYCN tumors. Surprisingly, intratumoral Tregs constituted a similar proportion of CD4+ T cells in early (8–20%) as in advanced tumors (6–16%; Figure 1D).

As a restriction element for peptide specific T cell responses, the expression of MHC class I in TH-MYCN tumors was investigated and low levels of the allele H-2Db in H-2D^d was detected in CD45+ cells (Figure 1E), whereas no expression of H-2Kb was detected in CD45− cells (Figure 1F).

Tumor-promoting cells of the innate immune system accumulate during tumor progression

The presence and phenotype of infiltrating TAMs (defined as dead cell stain/CX45+F4/80+, Supplementary Figure 1B, available at Carcinogenesis Online) in TH-MYCN tumors of different stages
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were evaluated by flow cytometry (Figure 2A). Tumors of all stages demonstrated detectable infiltration of TAMs, but in advanced tumors, TAMs constituted a significantly higher proportion of all infiltrating cells (Figure 2A). Furthermore, phenotypical evaluation of TAMs revealed an ongoing transition from an antitumor M1 to a tumor-promoting M2 phenotype in the course of tumor growth (Figure 2A). In small tumor lesions, TAMs displayed high expression of MHC class II (I-A^d, 65% median expression) and a tendency of lower expression of CD206 (macrophage mannose receptor, MMR; Figure 2A), in accordance with the phenotype of M1 macrophages (23, 24). In contrast, the opposite phenotype was detected at later stages of tumor development (I-A^d, 35% median positivity, Figure 2A).

Similar to TAMs, immature dendritic cells (DCs) are believed to possess the ability to promote tumor growth (25). Therefore, the presence and phenotype of infiltrating DCs (defined as dead cell stain^CD45^-CD11c^, Supplementary Figure 1B, available at Carcinogenesis Online) were investigated in the course of NB tumor growth using flow cytometry. DCs were present in all investigated stages of disease and constituted an expanding proportion of the infiltrating hematopoietic compartment (Figure 2B). Phenotypically, intratumoral DCs exhibited a dynamic pattern of MHC class II expression, with a reduced level of expression in advanced tumors (Figure 2B), indicative of an immature phenotype (25).

The presence of MDSCs (defined as dead cell stain^CD45^-Gr1^-CD11b^, Supplementary Figure 1B, available at Carcinogenesis Online), known for their versatile abilities to inhibit T cell-mediated antitumor immunity (15), was investigated. Using flow cytometry, an accumulation of MDSCs was detected in TH-MYCN tumors during progression (Figure 2C). Infiltrating hematopoietic cells represented an average 35% of all alive cells in the tumor mass, in smaller lesions and in larger tumors (Supplementary Figure 1A, available at Carcinogenesis Online). Hence, the actual number of hematopoietic cells increased in parallel with tumor progression, indicating an influx of innate cells into the tumor.

**T**2 cytokines dominate the TH-MYCN microenvironment throughout tumor development

Using quantitative real-time RT-PCR, we investigated the expression of interleukin (IL)-2, interferon (IFN)-γ, IL-6, IL-10 and transforming growth factor (TGF)-β (relative to the housekeeping gene hypoxanthine phosphoribosyltransferase, HPRT) in the course of neoplastic progression in TH-MYCN tumors. Strikingly, Th2 cytokines (IL-10 and TGF-β) dominated the tumor microenvironment and the expression pattern was stable during tumor progression. When comparing heterozygous and homozygous tumors of various sizes, heterozygous tumors exhibited a significantly higher relative expression of IL-10 and TGF-β (Figure 3B).

Low-dose aspirin reduces tumor burden in homozygous TH-MYCN mice and affects tumor-associated inflammation

The expression of COX-1, implicated in the production of several inflammatory mediators, was investigated using immunohistochemistry and flow cytometry, and high expression was detected in the majority of tumor cells (flow cytometry: CD45) from 5 week old homozygous mice (Figure 4A). Subsequently, 10 mg/kg aspirin (corresponding to approximately 60 mg in an adult human) (26) was administered once daily by gavage for 10 days to homozygous mice starting at the age of 4.5 weeks and proceeded until the mice were sacrificed at the age of 6 weeks. In contrast to corresponding untreated homozygous tumors, the majority of aspirin-treated tumors did not displace adjacent kidneys (Figure 4C) and the median tumor burden was significantly lower in aspirin-treated animals (0.25 g) than in control animals (1.15 g; Figure 4B).

Interestingly, the tumor-associated hematopoietic compartment in aspirin-treated tumors displayed a reduced proportion of innate cells such as MDSCs, DCs and TAMs (Figure 4D). Analysis of cytokine expression revealed significantly lower levels of TGF-β in treated
tumors (Figure 4E), whereas no significant difference was detected when analyzing IL-2, IFN-γ, IL-10 and IL-6 (Supplementary Figure 2, available at Carcinogenesis Online). Furthermore, the total amount of COX metabolites taken together (PGE2, TXA2, as analyzed through the stable metabolite TXB2, PGD2, 6-keto-PGF1α and PGF2α) was not statistically different in aspirin-treated tumors versus control tumors (data not shown). However, when analyzing the COX metabolites separately, a significant reduction in both TXA2 and PGD2 was revealed in the treated tumors. The median concentration of TXA2 and PGD2 in tumors treated with low-dose aspirin was 58% and 40%, respectively, of that of the non-treated controls (Figure 4F and G).

Discussion

During the last decade, the concept of cancer-related inflammation and the notion that tumor-infiltrating cells of the immune system may support tumor progression has challenged our view of antitumor immunity. Possessing an intrinsic ability to eradicate tumors, the immune system may still tip the balance toward cancer-related inflammation, as has recently been suggested to be incorporated as an emerging hallmark of cancer (3). The protumorigenic properties elicited by chronic inflammatory conditions have been attributed to the direct stimulation of tumor growth, as well as to the suppression of adaptive tumor immunity and to the promotion of populations with inhibiting functions such as Tregs, TAMs and MDSCs (15,27,28).

Subsequently, cancer-related inflammation portrays itself as a potential target in modern cancer therapy. Recently, Rothwell et al. published convincing data that the incidence of a number of adult cancer types was decreased in populations reporting daily intake of aspirin. Furthermore, low doses were as effective as high doses, minimizing the risk of unwanted side effects (6–9). Besides, it was shown that low-dose aspirin reduced further development of adenomas in patients diagnosed with colorectal cancer (29) and that disease-specific mortality was reduced in colorectal cancer and breast cancer patients using aspirin on a regular basis postdiagnosis (30,31). Previous studies have evaluated aspirin at low doses on other tumors (32), but not at doses as low as in our study, and no studies have evaluated aspirin on established NB.

Because most epidemiological and experimental studies addressing the role of inflammation in tumor development originate in adult models, we sought to investigate the contribution of cancer-related inflammation and the in vivo effect of low-dose aspirin on established tumors in the TH-MYCN transgenic mouse model of NB, which is the most common extracranial solid tumor in early childhood (33,34).
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The model represents a unique platform for monitoring the plasticity of the tumor microenvironment without introducing artificial components as is inevitable in the case of syngeneic or xenograft mouse models (17,18). In homozygous mice, hyperplasia has been described to occur in abdominal ganglia already during the first week of life (17), enabling early studies of tumor-associated changes in the microenvironment.

Our present study has investigated the presence, magnitude and composition of inflammation accompanying NB tumor growth in the TH- MYCN transgenic mouse model. In contrast to data published previously (22), we could detect tumor-infiltrating lymphocytes in all screened tumors, including early tumor lesions from homozygous mice at the age of 5 weeks using both immunohistochemistry and flow cytometry (Figure 1A and B). The underlying reason for this discrepancy may be that previous data neither relied on specific hematopoietic markers nor applied more than one technology. Furthermore, we monitored the dynamics of the hematopoietic compartment during tumor progression. The percentage of CD45+ cells as a part of all alive cells within the tumor mass neither increased nor decreased as tumors expanded (average 35%), indicating that the absolute number of present CD45+ cells increased during tumor progression (Supplementary Figure 1A, available at Carcinogenesis Online). Strikingly, components of an early adaptive immune response were replaced during tumor progression by cells of the innate immune system such as TAMs, immature DCs and MDSCs. In early tumor lesions, CD3+ T cells constituted a prominent part of all infiltrating cells and CD8+ T cells were readily detectable. So far, the presence of T cell antigens has not been investigated in the TH- MYCN model, but in humans, peptides derived from the MYCN protein are able to induce cytotoxic T cell responses from NB patients (35,36), indicating that tolerance to MYCN may be broken, and this would therefore be of interest to investigate in TH- MYCN tumors. However, the proportion of T cells, as well as the fraction of CD8+ T cells within the T cell compartment, was diminished in parallel to tumor growth (Figure 1B and C). CD4+ T-helper cells, which constituted the predominant subtype of T cells in TH- MYCN tumors, have been ascribed paradoxical roles in human tumors, with correlation to improved clinical outcome in lung cancer (37) and the inverse relationship in other models (38,39). Proposed underlying explanations for this duality include the T H 1/T H 2 dogma, with T H 1 cells promoting immunosurveillance and T H 2 cells dampening cytotoxic T-lymphocyte responses and/or stimulating tumor growth (40). Furthermore, immunosuppressive and tumor-promoting abilities conferred by CD4+ T cells are often mediated by CD4+ FOXP3+ Tregs (27), and their intratumoral presence is known to be a poor prognostic factor in many cancers (38,41). In human non- MYCN amplified stage 4 NB tumors, the expression of FOXP3 did not significantly correlate to prognosis, yet fewer patients with FOXP3 expression above median

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had a 5 year event-free survival (42). We detected infiltrating Tregs in all screened TH-MYCN tumors. Surprisingly, their prevalence within the CD4+ compartment was steady and did not increase in the course of tumor growth (Figure 1D).

During cancer progression, components of the innate immune system, such as MDSCs, DCs and TAMs, are educated by the tumor to promote its growth and obtain an immature phenotype (25,28,42–44). Concurrently to the observed suppression of T cell responses, TAMs, MDSCs and DCs represented an expanding proportion of the hematopoietic compartment (Figure 2). When evaluating TAMs, we could demonstrate a gradual transition from an M1 phenotype (MMRlow/MHC class IIlow) in early tumor lesions to an M2 phenotype (MMRhigh/MHC class IImod) at later stages of tumor development (Figure 2A). This sheds new light on data published by Song et al., showing that natural killer T cells mediate NB antitumor immunity by killing Cd1D expressing TAMs and that monocyte/macrophage-related genes such as CD14 and CD16 correlated to poor prognosis in stage 4 non-MYCN amplified NBs (42). Although DCs were reported to be sparse within pediatric tumors (45), the TH-MYCN tumors were populated with infiltrating DCs that displayed reduced levels of MHC class II during tumor progression (Figure 2B). This finding suggests the presence and accumulation of immature and disarmed DCs in TH-MYCN NB tumors. Furthermore, MDSCs, known for their broad repertoire of immunosuppressive mechanisms, accumulated in tumors during progression (Figure 2C), again disclosing a gradual onset of an unfavorable inflammatory composition. These data are in

**Fig. 4.** Expression of COX-1 and anti-inflammatory treatment with low-dose aspirin. (A) Expression of COX-1 in TH-MYCN tumors detected by flow cytometry in CD45+ cells (solid black line, 63% positivity) and CD45− cells (solid gray line, 95% positivity) compared with control staining (filled histogram), and by immunohistochemistry. (B) Tumor weights of 5 week old control mice (n = 8), 6 week old control mice (n = 15) and 6 week old aspirin-treated mice (n = 8). (C) Visualization of control and aspirin-treated tumors; T = tumor, K = kidney. Histological analysis showing HE staining of control and aspirin-treated tumors. (D) Graph representing flow cytometry data on infiltrating cells of the innate immune system as percentage of all infiltrating cells (dead cell stain/CD45+); open squares indicate Gr1+CD11b+ MDSCs (ncontrol = 7, nAspirin = 6), open circles represent CD11c+ DCs (ncontrol = 9, nAspirin = 4) and filled circles represent F4/80+ TAMs (ncontrol = 9, nAspirin = 6). (E) Relative expression of TGF-β as measured by quantitative RT–PCR in control versus aspirin-treated tumors. (F) The levels of TXA2 (as measured by analyzing the stable metabolite TXB2) given as percentage of the mean of control tumors (ncontrol = 10, nAspirin = 5). (G) The levels of PGD2 given as percentage of the mean of control tumors (ncontrol = 10, nAspirin = 5). Two-sided Mann–Whitney U-test was used for all statistical comparison among groups, *indicates P < 0.05, **indicates P < 0.01, ***indicates P < 0.001 and bars indicate median value.
line with a recent study demonstrating higher infiltration of TAMs in metastatic NBs than in locoregional tumors and higher expression of inflammation-related genes in metastatic tumors of patients diagnosed after 18 months of age (46). Another study concluded that a more active adaptive immune response prevails in high-risk NBs, whereas the innate immune system predominated in low-risk patients. The data might appear to contradict our results but the study is limited by few patient samples, no studies on intratumoral composition of immune cells and no data related to disease progression within risk groups (47).

In a permissive tumor microenvironment, antitumor responses are promoted by T_{H}1 cytokines such as IL-12, IL-2 and IFN-γ, whereas T_{H}2 cytokines such as IL-4, IL-6, IL-10 and TGF-β in general coincide with tumor promotion and immune escape (1,14). NB tumors in the TH-MYC_N model were accompanied by a T_{H}2-balanced microenvironment throughout the monitored time window, as defined by the predominance of IL-10 and TGF-β above IL-2 and IFN-γ (Figure 3A), thus strengthening the hypothesis of an early inflammatory drive participating in NB tumorigenesis and immunosuppression. Low levels of IL-6 were detected, which could possibly be explained by the negative regulation of IL-6 by MYCN (48). In fact, a correlation between high expression of IL-10, TGF-β and IL-6 and lower 5 year event-free survival in non-MYC_N-amplified NBs has previously been observed (42). Interestingly, heterozygous tumors, with a later onset and slower growth, displayed significantly higher levels of IL-10 and TGF-β, possibly reflecting a chronic inflammatory microenvironment (Figure 3B).

The presence of inflammatory patterns led us to investigate whether the COX-1 pathway was present and could contribute to tumor progression. The COX axis provides several potential tumor-promoting features because prostaglandins such as PGE_2 have the ability to directly stimulate tumor growth, to dictate angiogenesis, to stimulate the production of pro-inflammatory components and to regulate the influx of immunosuppressive cells such as MDSCs and Tregs while inhibiting cytotoxic T-lymphocyte responses (49). Expression of COX-1 was detected in CD45⁻ and in CD45⁺ cells in early tumor lesions (Figure 4A). Whereas COX-2 overexpression in tumor cells is well established for several cancers, including NB(16), COX-1, which is considered to be constitutively expressed, has also been reported to be overexpressed in ovarian, as well as in head and neck cancers (50,51).

Above mentioned data suggests that cancer-related inflammation is a potential target also in pediatric cancers such as NB. Hence, we investigated whether treatment with low-dose aspirin for 10 consecutive days, starting at the age of 4.5 weeks when tumors first appeared visible by eye, could affect tumor outgrowth in homozgyous TH-MYC_N mice. At the age of 6 weeks, aspirin-treated animals presented with a significantly lower tumor burden compared with untreated counterparts (Figure 4B). Interestingly, aspirin-treated tumors displayed a lower frequency of tumor-associated cells from the innate immune system including MDSCs, immature DCs and TAMs, an observation that might be due to aspirin per se, or secondary due to the reduction in tumor volume (Figure 4D). Concomitantly, analyzing the concentrations of extracted COX metabolites in treated versus untreated tumors revealed a significant decrease in the levels of TXA₂ (as measured by analyzing TXB₂) and PGD₂ (Figure 4F and G). Recently, the tumor growth-promoting role of PGE₂ in NB was shown (52), whereas the significance of reduced levels of PGD₂ remains unknown. Low doses of aspirin are known to irreversibly inactivate COX-1 in platelets and reduce platelet activation by diminished levels of TXA₂. Platelet activation has been suggested to be involved in the early stages of colorectal carcinogenesis, as well as in the later stages by mediating metastases formation. Aspirin-induced reduction of TXA₂ has also been suggested to reduce neoplasia by downregulation of COX-2 and by reducing angiogenesis (53,54). Furthermore, TXA₂ receptor signaling mediates recruitment of macrophages on liver damage (55), indicating a plausible connection between the reduction in TXA₂ levels and the reduced levels of innate cells, including macrophages, observed by us. Moreover, although the biological consequence remains to be studied, treated tumors displayed significantly lower expression of the immunosuppressive cytokine TGF-β (Figure 4E). To our knowledge, this represents the first in vivo data on how low-dose aspirin affects the composition of the tumor microenvironment and unveils a potential mechanism underlying its antitumor effects. Additional studies are warranted to delineate how low-dose aspirin affects the function of tumor-infiltrating hematopoietic cells at different stages of tumorigenesis.

In conclusion, our present study demonstrates that tumor-associated inflammation is a potential therapeutic target in the pediatric neoplasm NB. It also provides in vivo data in the aggressive TH-MYC_N model of NB demonstrating that low-dose aspirin as a single agent may inhibit tumor growth and affect the tumor microenvironment. Hence, low-dose aspirin should be further evaluated as an auxiliary treatment for high-risk NB tumors with MYCN amplification.

Supplementary material
Supplementary Table 1 and Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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References


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