Inhibition of Aurora-A Promotes CD8+ T-Cell Infiltration by Mediating IL10 Production in Cancer Cells

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ABSTRACT

Intratumoral tumor-specific activated CD8+ T cells with functions in antitumor immune surveillance predict metastasis and clinical outcome in human colorectal cancer. Intratumoral CD8+ T cells also affect treatment with immune checkpoint inhibitors. Interestingly, inhibition of Aurora kinase A (Aurora-A) by its selective inhibitor alisertib obviously induced infiltration of CD8+ T cells. However, the mechanisms by which inhibition of Aurora-A promotes infiltration of intratumoral CD8+ T cells remain unclear. Our recent results demonstrated that conditional deletion of the AURKA gene or blockade of Aurora-A by alisertib slowed tumor growth in association with an increase in the infiltration of intratumoral CD8+ T cells as well as the mRNA levels of their IL10 receptor α (IL10Rα). The antitumor effects of targeting Aurora-A were attenuated in the absence of CD8+ T cells. In addition, antibody-mediated blockade of IL10Rx dramatically decreased the percentage of intratumoral CD8+ T cells. In further experiments, we found that the levels of IL10 were elevated in the serum of xenografts treated with alisertib. Moreover, mice deficient in the IL10α receptor (VillinCre/ox/) and expressing a PEGylated IL10 (PEGIL10) demonstrated improved prognosis for patients (7). Unfortunately, recent studies have found that tumor clearance was enhanced in the presence of IL10 (18, 19). In addition, elevation of IL10 in experimental tumors potentiated the antitumor effects of tumor-specific CD8+ T cells (14, 20–23). Importantly, recent studies have demonstrated that PEGylated IL10 promoted the expansion of tumor-specific CD8+ T cells and enhanced polyclonal T-cell expansion in patients with cancer (9, 20, 21). All of these observations suggested that inducing IL10 secretion could be a useful treatment strategy.

Introduction

The extent of intratumoral CD8+ T cells is predictive of the overall survival of individuals with colorectal cancer (1–6), suggesting that the infiltration and activation of intratumoral CD8+ T cells results in an improved prognosis for patients (7–9). Unfortunately, recent studies have shown that only approximately 10% of intratumoral CD8+ T cells are tumor-specific CD8+ T cells (10). Moreover, multiple studies have demonstrated systemic activation of the T-cell repertoire in parallel with dose-limiting autoimmunity, although immunotherapy with multiple immune checkpoint inhibitors improved the clinical responses (11, 12). These findings indicated that elucidating the underlying mechanisms and developing strategies to recruit, activate, and expand tumor-specific intratumoral CD8+ T cells are urgently needed.

The capacity of migration and activation of CD8+ T cells is governed in a complex manner by surface expression of chemokine receptors and specific ligands (13). Several cytokines, such as IL10, have been shown to be critical for CD8+ T-cell migration and activation. IL10 is well known for its anti-inflammatory function mediated by inhibiting the secretion of proinflammatory cytokines and suppressing the expression of major histocompatibility complex (MHC) molecules as well as costimulatory molecules (14, 15). However, mice and humans deficient in IL10 or the IL-10 receptor develop inflammatory bowel disease and cancer (16, 17). Interestingly, recent studies have found that tumor clearance was enhanced in the presence of IL10 (18, 19). In addition, elevation of IL10 in experimental tumors potentiated the antitumor effects of tumor-specific CD8+ T cells (14, 20–23). Importantly, recent studies have demonstrated that PEGylated IL10 promoted the expansion of tumor-specific CD8+ T cells and enhanced polyclonal T-cell expansion in patients with cancer (9, 20, 21). All of these observations suggested that inducing IL10 secretion could be a useful treatment strategy.

Aurora-A, one of the members of the serine/threonine kinase family, functions as a "mitosis sensor" and is aberrantly expressed in various types of cancers, including colorectal cancer and hematopoietic malignancies (24, 25). During antigen-driven T-cell activation, Aurora-A was shown to be phosphorylated and recruited to the immunological synapse (IS). Either deletion of AURKA or pharmacological inhibition of Aurora-A function reduced the accumulation of phosphorylated Aurora-A at the IS and impeded the movement of vesicles towards the IS structure but did not result in a global defect in cytoskeleton dynamics (26), indicating that Aurora-A can be involved in controlling the immune response. Treatment of a melanoma xenograft model with alisertib, an oral Aurora-A selective inhibitor, enhanced the infiltration of helper and cytotoxic T cells into tumors (27), indicating that inhibition of Aurora-A could promote infiltration of intratumoral CD8+ T cells. However, the mechanisms by which blockade of Aurora-A promote tumor-infiltrating lymphocytes (TIL) may not be related to the...
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critical role of Aurora-A in antigen-driven T-cell activation. A previous study showed that nuclear Aurora-A interacted with heterogeneous nuclear ribonucleoprotein K (hnRNP K) and functioned as a transcription factor (28). To investigate whether inhibition of Aurora-A promoted CD8⁺ T-cell infiltration, we utilized the CT-26 mouse model and the azoxymethane (AOM)/dextran sodium sulfate (DSS) colitis-associated model of colon cancer. We found that blockade of Aurora-A induced infiltration of CD8⁺ T cells through elevated IL10, at least partially.

Materials and Methods

Reagents

Aisertib (S1133) was purchased from Selleck Chemicals. The antibody for IFNγ (505809), an in vivo anti-CD8 antibody (100701) and an in vivo anti-IL10R antibody (112711) were purchased from Biolegend. Antibodies against CD45 (550994), CD3 (553061), CD8 (552877), CD19 (560375), CD11b (557657), CD206 (565250), and F4/80 (565410) were purchased from BD Biosciences. Recombinant mouse IL10 (ab04067) was purchased from Absin.

Isolation of intestinal epithelial cells, cell lines, and cell culture

The crypt cells were isolated as described previously (29). Briefly, the intestinal sections were incubated in precooled Hank's balanced salt solution (HBSS; Gibco, Invitrogen) containing antibiotics at room temperature for 15 minutes and then cut into 0.5 cm pieces. Crypt epithelial cells were dissociated by repeated vigorous shaking and collected by centrifugation at 200 x g for 15 minutes at 4°C. Cell pellets were resuspended in 10 mL of HBSS containing 0.4 mg/mL dispase (Life Technologies), 10,000 U/mL penicillin/streptomycin, and 5% FBS. After 30 minutes of incubation at 37°C, the cells were collected by centrifugation at 150 x g for 10 minutes at 4°C.

Murine CT-26 cells were purchased from ATCC and grown in RPMI1640 containing 10% heat-inactivated FBS. Murine MC-38 cells and human SW480 cells were purchased from FuHeng (FuHeng Cell Center) and maintained in DMEM supplemented with 10% FBS. The identities of the cell lines were verified by short tandem repeat analysis. All cell lines were used in their early passages and routinely tested for mycoplasma contamination by Mycoplasma Test Kit (Clark Bioscience, T102).

Generation of the mice with conditional AURKA knockout

For generation of the mice with conditional knockouT of AURKA, an 8.8-kb mouse AURKA gene-containing fragment was subcloned into the plucoselect II SK vector and was used to create an AURKA loxP targeting vector. This vector was constructed by inserting an FRT-PGK-Neo-FRT-loxP cassette into intron 3 and another loxP site into intron 4, followed by an HSV-TK expression cassette at the 3’ end of the construct to be used as a negative selection marker. For excision of the neomycin selection marker, the transgenic mice were mated with Ella-Cre transgenic mice (The Jackson Laboratory, stock number 003724), followed by Cre-mediated deletion of the neomycin selection marker, the transgenic mice were bred and maintained under protocols approved by the Animal Institutional Care and Use Committee at Xuzhou Medical University.

Model of colorectal cancer associated with colitis

Colorectal cancer associated with colitis was induced as described previously (29, 30). Briefly, 12- to 14-week-old mice were intraperitoneal injected with 10 mg/kg AOM (Sigma-Aldrich), followed by treatment with 1% DSS (MW 36,000–50,000 Da; MP Biomedicals) in drinking water for seven consecutive days. Then, the mice were administered normal drinking water for 14 days. This DSS treatment was repeated for three additional cycles. On day 63 of the regime, the mice were euthanized. The intestinal and colon sections were removed, washed with PBS, and opened longitudinally for analysis.

Ectopic tumor implantation and weight measurements

The mouse strains were maintained and housed under specific pathogen-free (SPF) conditions. The tumor experiments used 6-week-old female Balb/c mice. The left and right flanks of the mice were shaved, and CT-26 cells (1 x 10⁶) were injected subcutaneously. When palpable tumors formed, the mice were randomized to receive either 20 mg/kg alisertib (20 mg/kg) orally for 14 consecutive days. The tumor size was measured three times a week using calipers. The tumor sizes were calculated using the formula a x b x c, where “a” is the length, “b” is the width, and “c” is the height in millimeters. At the end of the experiment, the animals were killed by CO₂ asphyxiation, and the tumor weights were measured after careful resection. The tumor tissues were collected for further analysis.

For CD8⁺ cell depletion studies, anti-CD8 (clone 53-6.7) antibody at 0.35 mg per mouse (Biolegend) was administered 4 days and 1 day prior to initiation of dosing with alisertib and every 7 days thereafter. The rat IgG2a κ isotype control antibody clone Rtk2758 (Biolegend) was used as a negative control. The animals were not dosed with alisertib on the days they received the antibodies to limit anti-antibody responses. Depletion of CD8⁺ T cells was confirmed by flow cytometry on days 8 and 13.

For the Rag2⁻/⁻ mice in vivo, wild-type C57BL/6 and Rag2⁻/⁻ mice were a gift from Hui Wang (Xuzhou Medical University). A total of 1 x 10⁶ MC-38 cells were injected subcutaneously into the left flank of 8- to 12-week-old wild-type or Rag2⁻/⁻ mice. When palpable tumors formed, the mice were administered either a drug diluent alone (control) or alisertib (20 mg/kg) orally every day. The tumor size was monitored every other day. After 14 days, the animals were killed by CO₂ asphyxiation, and the tumors were isolated to determine the weight following careful dissection.

For the blocking IL10R studies in vivo, ultralow endotoxin, azide-free purified blocking antibody against the murine IL10 receptor (IL10R; CD210) (et-IL10R)-clone 1B1.3a or mouse IgG2a, κ isotype control antibody clone MOPC-173 (Biolegend) was administered intraperitoneally six times at a dose of 0.25 mg of antibody to the mice on days 0, 1, 2, 5, 8, and 11. During the anti-IL10Rt antibody treatment, alisertib was administered orally every day.

Tumor digestion and preparation of single cell suspensions

For TIL isolation, the tumors were removed and manually dissociated. The cells were washed through 70 μm filters, washed twice and analyzed by flow cytometry. Suspensions of spleen cells were obtained by mashing the spleen through a 70-μm nylon cell strainer (BD Falcon).

Surface staining, intracellular staining, flow cytometry, and cell sorting

Surface staining was performed with mAbs for 30 minutes at 4°C in PBS using the indicated antibodies listed. For intranuclear staining, the cells were first stained at the surface before fixation.
AURKA Inhibition Enhances CD8⁺ TIL Abundance

Figure 1.
Inhibition of Aurora-A activity by alisertib suppressed tumor growth by promoting infiltration and expansion of intratumoral CD8⁺ T cells. A, Growth curve of syngeneic mouse tumors with the control treatment (n = 5, in black) or the alisertib treatment (n = 5, in red). Data shown are representative of one of three independent experiments with n = 5 mice/group (P < 0.05). B, Tumors were removed and weighed at the end of the experiment (n = 8-10 tumors/group). The results represent the mean ± SEM of the tumor weights (P < 0.05). C, Body weight of the mice was measured twice a week during treatment. Data shown are representative of one of three independent experiments with n = 5 mice/group (n.s., not significant). D, The population of intratumoral CD8⁺ T cells, IFNγ⁺ CD8⁺ T cells and exhausted CD8⁺ T cells was investigated by FACS. Data shown are representative of one of three independent experiments with n = 5 mice/group (P < 0.05). E, Immunofluorescence for CD8 (green), TIM-3 (red), PD-1 (cyan), IFNγ (yellow), Ki-67 (purple), and DAPI (blue) in the tumor sections treated with or without alisertib. Data shown are representative of one of two independent experiments with n = 3 tumor sections/group. F-I, Quantitation of CD8⁺ IFNγ⁺ T cells (F), CD8⁺ PD-1⁺ Ki-67⁺ T cells (G), CD8⁺ TIM-3⁺ Ki-67⁺ T cells (H) in the tumor sections treated with or without alisertib. At least 10 representative fields were quantified per section. Data shown are representative of one of two independent experiments with n = 3 tumor sections/group. The results represent the mean ± SEM (P < 0.05). I, The population of IFNγ⁺ CD8⁺ T cells in the spleen was examined by FACS; data shown are representative of one of three independent experiments with n = 5/group (n.s., not significant).
Figure 2.
The antitumor ability of alisertib was attenuated in the absence of T cells. A, Approximately 1 x 10^6 mouse colon adenocarcinoma MC38 cells were injected subcutaneously into the flanks of 6- to 8-week-old C57BL/6 mice. The mice were treated with the control (n = 5), alisertib (n = 5), an anti-CD8 antibody (n = 5) or alisertib in combination with the anti-CD8 antibody (n = 5). The population of CD8^+ T cells in the peripheral blood of the mice treated with the isotype control or the antibody against CD8 was examined by FACS on day 8 and day 13 (n = 5/group). Data are representative of one of two independent experiments. The results represent the mean ± SEM of the percentage of CD8^+ T cells (*, P < 0.01). B, Tumor volumes were recorded in the MC38 syngeneic mouse model. Data are representative of one of two independent experiments with n = 5 mice/group (*, P < 0.05; n.s., not significant). C, At the end of the treatment, the tumors were removed and weighed. Data are representative of one of two independent experiments with n = 8 to 10 tumors/group. The results represent the mean ± SEM of tumor weights (*, P < 0.01). D, The population of intratumoral CD8^+ T cells was examined by FACS. Data shown are representative of one of two independent experiments with n = 8 to 10 tumors/group. The results represent the mean ± SEM of CD8^+ T cells (%) (*, P < 0.05; n.s., not significant). E, Growth curve of transplanted tumors treated with the control or alisertib in WT (solid lines) and Rag2^−/− (dashed lines) mice. Data are representative of one of two independent experiments with n = 5 mice/group. The results represent the mean ± SEM of tumor volume (*, P < 0.05; n.s., not significant). F, At the end of the treatment, the tumors were removed and weighed. Data are representative of one of two independent experiments with n = 7 to 10 tumors/group. The results represent the mean ± SEM of tumor weights (*, P < 0.01; n.s., not significant).
AURKA Inhibition Enhances CD8⁺ TIL Abundance

**Figure 3.**
Conditional knockout of AURKA inhibited tumor development by promoting the percentage of intratumoral CD8⁺ T cells. 

A, Schematic representation of the AURKA targeting vector. Thick numbered boxes represent the exons. Triangles indicate the presence and orientation of the loxP sites. B, PCR amplification of genomic DNA isolated from the toe snips of mouse pups. C, Real-time RT-PCR to quantify the levels of Aurora-A mRNA in crypt cells isolated from either the AURKA⁺/⁺;VilCre⁺ mice (n = 3/group). Data are representative of one of three independent experiments performed in duplicate (*, P < 0.05). D, Representative photomicrographs of the H&E-stained sections of the intestine and colon of 6- to 8-week-old AURKA⁺/⁺ and AURKA⁻/⁻;VilCre⁺ mice (n = 3). Data shown are representative of one of two independent experiments with n = 4 mice/group. E, The number of CD8⁺ T cells in the intestine and colon tissues from the AURKA⁺/⁺;VilCre⁺ and AURKA⁻/⁻ mice was counted under a microscope. Data shown are representative of one of two independent experiments with n = 4 mice/group. Data are representative of one of two independent experiments with n = 4 mice/group. F, The number of CD8⁺ T cells in the intestine and colon tissues from the AURKA⁺/⁺;VilCre⁺ and AURKA⁻/⁻ mice was counted under a microscope. Data shown are representative of one of two independent experiments with n = 4 mice/group. G, Tumor incidence in the AURKA⁺/⁺ (n = 7) and AURKA⁻/⁻;VilCre⁺ mice (n = 11) after AOM/DSS treatment. Data are representative of one of two independent experiments. H, Tumor number (left) and tumor volume (right) comparison between the AURKA⁺/⁺ (n = 7) and AURKA⁻/⁻;VilCre⁺ mice (n = 11). Data are representative of one of two independent experiments. I, Immunofluorescence for CD8 (red) and DAPI (blue) in the tumor tissue sections from the AURKA⁺/⁺ or AURKA⁻/⁻;VilCre⁺ mice. The number of CD8⁺ T cells was counted in the tumor sections from the AURKA⁺/⁺ or AURKA⁻/⁻;VilCre⁺ mice (n = 5/group). Data are representative of one of two independent experiments. The results represent the mean ± SEM of the number of CD8⁺ T cells (*, P < 0.05).
Figure 4.

IL10Rx was required for alisertib-mediated infiltration of intratumoral CD8+ T cells. A, RNA was extracted from a total of 1 × 10^6 CD8+ T cells isolated from either control-treated or alisertib-treated tumors (n = 3/group) and subjected to real-time RT-PCR to detect the mRNA levels of cytokines and IL10Rx. Data are representative of one of three independent experiments performed in duplicate. Data are shown as the mean ± SEM and were compared using Student t test (*, P < 0.05). B, Approximately 1 × 10^6 mouse colon adenocarcinoma MC-38 cells were injected subcutaneously into the flanks of 6- to 8-week-old C57BL/6 mice. The mice were treated with the control, alisertib, anti-IL10Rx antibody or alisertib in combination with anti-IL-10Rx antibody. The tumor volumes were measured in an MC-38 syngeneic mouse model. Data are representative of one of two independent experiments with n = 5 mice/group (*, P < 0.05). C, Tumor weights at autopsy. Data are representative of one of two independent experiments with n = 8 to 10 tumors/group. (Continued on the following page.)
and permeabilization using a Transcription Factor Staining Kit (BD Biosciences) followed by intranuclear staining. Spleens, dLNs and tumors were isolated. Single cell suspensions were used for flow cytometry. Data were analyzed using FlowJo (FlowJo V10).

For cell sorting, CD8+ cells enriched from the TIL suspensions were stained with anti-CD3ε (BD Biosciences, 145–2C11), anti-CD8α (BD Biosciences, 53–67), and anti-CD45 (BD Biosciences, 30–F11). CD45–CD3–CD8+ cells were sorted on a FACSAria (BD Biosciences) flow cytometer. The infiltrating T cells (CD45+CD3+ cells) from the intestinal and colon tissues of Aurora-A conditional knockout mice were stained with anti-CD11b (BD Biosciences, M1/70) and anti-CD45 (BD Biosciences, 30–F11). CD45–CD11b+ cells were sorted on a FACSAria (BD Biosciences) flow cytometer. The purity of the sorted cells was greater than 99%.

**Real-time RT-PCR**

A total of 1 × 10^6 cells were lysed in lysis buffer (TaKaRa, 3735A) and subjected to real-time RT-PCR according to the instruments. The primer sets for PCR were shown in Supplementary Table S1.

**Histologic analysis**

Tissues were fixed in 4% formaldehyde and embedded in paraffin. Sections with a thickness of 4 μm were stained with hematoxylin–eosin (Beyotime Biotechnology) as described previously (29, 31).

**IHC**

IHC staining was performed on formalin-fixed, paraffin-embedded tissue samples purchased from Shanghai Outdo Biotech. The levels of the immunostained tissues were evaluated independently by two pathologists who were blind to the subjects’ clinical information. Between 15 and 20 high-power fields were viewed. Criteria were developed for quantitating the immunoreactivities of Aurora-A and CD8 staining using a score range of 0 to +3, where 0 indicated no positive cell staining, +1 indicated less than 10% positive cell staining, +2 indicated 10% to 30% positive cell staining, and +3 indicated more than 30% positive cell staining. Similarly, the intensity of staining was also graded as +, +1, +2, or +3 as described previously (32).

**Multiplexed immunofluorescence staining and quantification**

Multiplex staining and multispectral imaging were performed to identify the cell subsets expressing CD8α, PD-1, TIM-3, IFNγ, and Ki-67 in the TME using a PANO 7-plex IHC Kit (catalog no. 0004100100; Panovue). Different primary antibodies were sequentially applied, followed by horseradish peroxidase-conjugated secondary antibody incubation and tyramide signal amplification. The slides were microwave heat-treated after each TSA operation. Nuclei were stained with 4′-6′-diamidino-2-phenylindole (DAPI; Sigma) after all the human antigens had been labelled.
CD8<sup>+</sup> T cells were critical for alisertib-mediated tumor suppression

To assess whether CD8<sup>+</sup> T cells were essential for the alisertib-mediated tumor suppression, we treated the mice with anti-CD8 antibodies, which led to significant depletion of CD8<sup>+</sup> T cells in the peripheral blood (Fig. 2A). Importantly, depletion of the CD8<sup>+</sup> T cells significantly promoted the tumor growth and the tumor weight in association with a decrease in the population of intratumoral CD8<sup>+</sup> T cells in the presence of alisertib (Fig. 2B-D). To further investigate whether the antitumor effect of alisertib requires immune function, we established syngeneic tumor models by injection of the mouse colon adenocarcinoma cell line MC-38 into age-matched 6- to 8-week-old wild-type C57BL/6 or immunodeficient Rag<sup>2</sup>−/− mice that lack T cells. We found that alisertib did not suppress tumor growth in the Rag<sup>2</sup>−/− mice compared with the wild-type mice (Fig. 2E and F), suggesting that the antitumor effect of alisertib was dependent on the extent of infiltration of intratumoral CD8<sup>+</sup> T cells at least partially.

Deletion of Aurora-A decreased the colitis-associated tumor incidence in parallel with an increase in the number of intratumoral CD8<sup>+</sup> T cells

Aurora-A is required for early embryonic development, and AURKA knockout leads to early embryonic lethality (36). To avoid off-target effects and investigate the role of Aurora-A in promoting infiltration of CD8<sup>+</sup> T cells, we generated mice with Aurora-A conditional knockout (CKO) by crossing AURKA<sup>fl/fl</sup>/VilCre<sup>+</sup> (AURKA<sup>−/−</sup>) mice (Fig. 3A) with VilCre<sup>+</sup> (VilCre<sup>+</sup>) mice, which induced Cre-dependent recombination in the intestinal epithelium by embryonic day (E) 12.5. Villin expression was also found in the primitive endoderm, gut, nephron anlagen and developing embryo (37, 38).

A lethal phenotype was observed after intercrossing AURKA<sup>−/−</sup>;VilCre<sup>+</sup> with AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice (data not shown). We therefore utilized the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice. The specific deletion of AURKA alleles in adult AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice was determined by PCR analysis of genomic DNA (Fig. 3B). As shown in Fig. 3C, the Aurora-A mRNA levels were obviously decreased in crypt cells isolated from the intestines of the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice compared with those from the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice. Analyses of the intestine and colon sections from the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> and the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice at 6 weeks showed that the number of CD8<sup>+</sup> T cells was reduced in the intestine and colon tissues from the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice compared with that of AURKA<sup>−/−</sup> mice (Fig. 3D-F). To determine if the deletion had any effects later in development, we established a cohort of male and female mice and monitored them weekly for weight, activity and overt signs of disease. Both the male and female heterozygous deletion mice appeared to be identical to their AURKA<sup>−/−</sup> counterparts (data not shown). The AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice did not demonstrate any notable differences in disease occurrence, spontaneous tumor formation, or mortality compared with the AURKA<sup>−/−</sup> mice (data not shown).

To further study whether deletion of AURKA also contributed to recruitment of intratumoral CD8<sup>+</sup> T cell infiltration, we treated the AOM/DSS-treated mice with a combination of Cisplatin or DSS. The weight loss and AOM/DSS-induced mortality were similar in the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> and AURKA<sup>−/−</sup> mice (data not shown).

At the end of the experiments, the mice were euthanized. The tumor incidence was 100% in the AURKA<sup>−/−</sup> mice. However, only 6 of 11 AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice treated with AOM/DSS developed tumors located predominantly in the distal to middle colon (Fig. 3G). Compared with those of the AURKA<sup>−/−</sup> mice, the number of tumors and the size of the tumors were substantially decreased in the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice (Fig. 3H). Importantly, the population of intratumoral CD8<sup>+</sup> T cells was strongly elevated in the tumors of the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice (Fig. 3I). To further test whether CD8<sup>+</sup> T cells are involved in alisertib-mediated tumor suppression, we treated the AOM/DSS mouse model with alisertib and found that upon alisertib treatment, the tumor sizes decreased along with an increase in the percentage of intratumoral CD8<sup>+</sup> T cells in the AOM/DSS-treated AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice compared with the AOM/DSS-treated AURKA<sup>−/−</sup> mice (Supplementary Figs. S1A and S1B). Consistent with the findings in the CT-26 mouse model, the number of CD8<sup>+</sup> T cells in the thymus and lymph nodes of the AURKA<sup>−/−</sup>;VilCre<sup>+</sup> mice was identical to that of AURKA<sup>−/−</sup> mice (data not shown), suggesting that inhibition
of Aurora-A not only suppressed colon tumor initiation but also suppressed colon tumor development by promoting infiltration of CD8^+ T cells.

Inhibition of Aurora-A–mediated infiltration of intratumoral CD8^+ T cells was dependent on induction of IL10Rx expression in these cells

To determine the mechanisms by which silencing Aurora-A by alisertib or deletion promoted infiltration of intratumoral CD8^+ T cells, we performed gene expression profiling of FACS-sorted intratumoral CD8^+ T cells. Real-time RT-PCR confirmed that the mRNA levels of IFNg, granzyme B (GzmB), and perforin (PFP), an essential player in the cytotoxic activity of cytotoxic CD8^+ T cells (39), were significantly increased in the infiltrated CD8^+ T cells isolated from the alisertib-treated tumors (Fig. 4A). Notably, the IL10Rx mRNA levels were obviously elevated in the CD8^+ T cells isolated from the alisertib-treated tumors compared with those of the control-treated tumors (Fig. 4A). IL10Rx-deficient children were inclined to develop B-cell lymphomas due to a lack of infiltration of cytotoxic T cells (17). We therefore investigated the role of IL10Rx in the alisertib-induced recruitment and activation of intratumoral CD8^+ T cells utilizing an IL10Rx-specific blocking antibody. As shown in Fig. 4B, blockade of IL10Rx attenuated the ability of alisertib to suppress tumor growth. In addition, compared with those of the alisertib-alone-treated mice, the tumor weights increased in parallel with a decrease in the population of infiltrated CD8^+ T cells in the presence of the anti-IL10Rx antibody and alisertib (Fig. 4C and D). All of these observations implicated IL10Rx as a key mediator in alisertib-induced infiltration of tumorspecific CD8^+ T cells.

In contrast to IL10RB, IL10Rx appears to exclusively bind IL10 (40). In addition, upon IL10 treatment, the cytotoxicity and expansion of tumor-specific CD8^+ T cells as well as the expression of IL10Rx on these cells were potently induced (9, 20), suggesting that IL10 could be involved in the alisertib-mediated infiltration of intratumoral CD8^+ T cells. To address whether elevated expression of IL10Rx in infiltrated CD8^+ T cells was a consequence of the induction of IL10 mediated by Aurora-A inhibition, we exposed freshly sorted CD8^+ T cells to IL10 and found that the IL10Rx mRNA levels were elevated in the presence of IL10 (Supplementary Fig. S2), suggesting that the upregulated expression of IL10Rx in CD8^+ T cells could be a consequence of the elevated IL10 production.

IL10 transcription and production were enhanced

To explore the role of Aurora-A in IL10 production, we examined the IL10 levels in the culture medium of the CT-26 cells isolated from either the control or alisertib-treated tumors. We found that the concentration of IL10 was obviously increased in the culture medium of the CT-26 cells isolated from the alisertib-treated mice compared with that of the CT-26 cells isolated from the control-treated mice (Fig. 4E). In addition, the mRNA levels of IL10 were enhanced in the alisertib-treated CT-26 cells compared with the control-treated CT-26 cells (Fig. 4F). Similarly, the IL10 mRNA levels were dramatically elevated in crypt cells but not the infiltrating T cells (CD3^+ T cells) or CD11b^+ cells enriched in myeloid-derived suppressor cells (MDSC) that express high levels of IL10 in colon tissue (41) isolated from the colon of AURKA^+ ;VilCre^+ mice compared with AURKA^+ ;VilCre^mice (Fig. 4G). The levels of IL10 in the serum of the AOM/DSS-treated AURKA^+/+ ;VilCre^+ mice were also increased compared with those of the AURKA^+ ;VilCre^+ mice, although IL10 could not be detected in the serum of both the AURKA^+ ;VilCre^+ and AURKA^+ ;VilCre^mice (Fig. 4H).

Suppression of Aurora-A induced IL10 transcription in a mitotic defect-independent manner

Given that IL10 facilitated the Aurora-A inhibition-induced CD8^+ T-cell infiltration, we further examined which subset of cells were the major source of IL10, as well as the mechanisms by which IL10 was affected. The number of CD19^+ B cells was reduced in the alisertib-treated tumors compared with the control-treated tumors (Supplementary Fig. S3A). Similarly, the population of F4/80^+ CD11b^+ CD206^+ cells was decreased in the tumors isolated from the mice that received alisertib (Supplementary Fig. S3B), indicating that upon alisertib treatment, cancer cells might contribute to elevated IL10.

To investigate whether cancer cells were involved in mediating IL10 transcription in the presence of alisertib, we treated CT-26 cells and SW480 cells with various concentrations (0–1,000 nmol/L) of alisertib for 24 hours. We found that a high dosage of alisertib potentially increased the IL10 mRNA levels (Fig. 5A and B). In addition, we treated these cells with 1,000 nmol/L alisertib for the indicated durations and found that alisertib induced upregulation of the IL10 mRNA level after exposure of these cells to alisertib for 24 hours (Fig. 5C and D). To further determine whether IL10 was induced as a consequence of mitotic delay and chromosome segregation defects, we treated these two cell lines with nocodazole, which blocked the mitosis process as early as 3 hours (42). We found that the IL10 mRNA levels were decreased after exposure of the CT-26 cells to nocodazole (Fig. 5E). However, the IL10 mRNA levels were almost identical to those in the untreated SW480 cells (Fig. 5F). Furthermore, upon alisertib treatment, the transcription levels of IL10 were enhanced (Fig. 5G), revealing that the expression of IL10 could be directly suppressed by Aurora-A rather than as a consequence of mitotic defects mediated by Aurora-A inhibition.

Elevated IL10 mediated by inhibition of Aurora-A promoted CD8^+ T-cell migration

To examine whether IL10 secreted by cancer cells indeed recruited tumor-specific CD8^+ T-cell infiltration, we performed a migration assay. The migrated CD8^+ T cells were barely detected when CD8^+ T cells were exposed to the medium harvested from the control-treated CT-26 cells. Nevertheless, the number of migrated CD8^+ T cells was potently elevated after exposure of these cells to the media from the alisertib-treated CT-26 cells and was decreased in the presence of an anti-IL10Rx antibody (Fig. 5H).

Constitutive expression of Aurora-A was correlated with a low density of CD8^+ T cells in colorectal cancer

To further clarify the clinical relevance of Aurora-A and intratumoral CD8^+ T cells in colorectal cancer, we examined the expression of Aurora-A as well as the density of CD8^+ T cells by IHC. We found that 12 of 25 samples highly expressed Aurora-A, whereas the densities of CD8^+ T cells in these samples were barely detected (Fig. 6A, left; Table 1). However, high densities of intratumoral CD8^+ T cells were observed in both the tumor tissues and mesenchyme with low expression of Aurora-A (Fig. 6A, right; Table 1). Moreover, the correlation of Aurora-A and CD8^+ T cells was determined using the GEPIA web tool. Our data indicated that the expression of Aurora-A was negatively correlated with the expression of CX3CR1 and FGFRBP1 (effector T cells; Fig. 6B). In addition, the expression of Aurora-A was negatively correlated with the expression of PDCD1 and GZMA (effector memory T cells; Fig. 6C). Interestingly, the expression of Aurora-A was negatively correlated with the expression of IL10 (Fig. 6D), indicating that inhibition of Aurora-A could elevate IL10.
Figure 6.
The level of Aurora-A was negatively correlated with IL10 expression and CD8^+ T cells. A, IHC staining for colon cancer tissues from clinical patients (n = 25). Samples in the top row were stained for Aurora-A, those in the middle and bottom rows were stained for CD8. Data are representative of one of two independent experiments. B and C, The correlation of Aurora-A and CD8^+ T cell-related genes analyzed by the GEPIA website tool. D, The correlation of Aurora-A level and IL10 expression analyzed by the GEPIA website tool. E, Schematic figure demonstrating the role of the IL10 pathway in the alisertib-mediated CD8^+ T-cell antitumor effects. Inhibition of Aurora-A by alisertib or gene deletion promotes the transcription of IL10 in colon cancer cells, enhancing IL10 secretion, leading to upregulation of the expression of IL10Rx in CD8^+ T cells as well as the activation of CD8^+ T cells, which in turn target tumor cells.
transcription and production, stimulating IL10Rx expression in intratumoral CD8+ T cells, which could activate its downstream signaling pathway and contribute to enhancements of the functional activity of intratumoral CD8+ T cells (Fig. 6E).

Discussion

The AURKA gene is an oncogene that is amplified and/or over-expressed in many tumors, including leukemia and breast and colorectal cancers. In addition, inhibition of Aurora-A leads to mitotic delays, severe chromosome congression and segregation defects, followed by cell death. Therefore, Aurora-A has become a therapeutic target for the treatment of various malignancies. Alisertib, an oral inhibitor of Aurora-A, has been investigated in clinical trials. Consistent with a previous study showing that alisertib promoted the infiltration of helper and cytotoxic T cells into melanoma and breast cancer tumors, respectively (27, 43), we found that the antitumor effect of alisertib depended on the recruitment of intratumoral CD8+ T cells (Fig. 5H). Elevated IL10 promoted CD8+ T-cell infiltration (Fig. 3), indicating that inhibition of Aurora-A could be a new approach for inducing recruitment of intratumoral CD8+ T cells, although a subset of intratumoral CD8+ T cells expressed coinhibitory molecules.

It has been reported that CD8+ T cells expressing coinhibitory receptors, including PD-1, TIM-3, CTLA-4, or LAG-3, are generally considered exhausted T cells with reduced cytotoxic and proliferative capacity (44). However, a recent study demonstrated that CD8+ T cells simultaneously expressing the PD-1, TIM-3, and LAG-3 coinhibitory receptors were also enriched in tumor-specific T cells (45). Interestingly, it has been shown that pegylodecakin, PEGylated IL10, induced expansion of and functional activity of exhausted CD8+ T cells in parallel with an increase in the expression of IL10Rx of these exhausted CD8+ T cells (9). In addition, the extent of expansion of the tumor-specific CD8+ T cells was correlated with the tumor response (9). Similarly, we found that inhibition of Aurora-A by alisertib dramatically induced recruitment and expansion of infiltrated CD8+ T cells in association with upregulation of the IL10Rx levels (Fig. 4A). However, the antitumor effect of alisertib was attenuated in the presence of an anti-IL10Rx antibody (Fig. 4B–D). In further experiments, we found that elevated expression of IL10Rx in the infiltrated CD8+ T cells could be a consequence of the induction of IL10-mediated by Aurora-A inhibition (Supplementary Fig S2). All of these data indicated that alisertib could promote infiltration of tumor-specific CD8+ T cells by controlling the expression of IL10Rx. Unlike IL10 Rβ, IL10Rx appears to exclusively bind IL10 (40). Our results demonstrated that the levels of IL10 were significantly elevated in the culture medium of the alisertib-treated CT-26 cells (Fig. 4E). In addition, the levels of IL10 were elevated in the serum of the AOM/DSS-treated AURKA+/−;VilCre−/− mice compared with the AURKA+/− mice, although IL10 could not be detected in the serum of both the AURKA+/−;VilCre−/− and AURKA+/− mice (Fig. 4H). Importantly, inhibition of Aurora-A could directly induce an increase in IL10 transcription, which was not a consequence of mitotic defects mediated by Aurora-A inhibition (Fig. 5A–G). Elevated IL10 promoted CD8+ T-cell migration (Fig. 5H). All of these observations indicated that elevated IL10 was involved in promoting CD8+ T-cell infiltration, although the mechanisms by which Aurora-A inhibited IL10 transcription need to be investigated in further experiments.

It has been reported that IL10 plays an important antiinflammatory role (9). However, a high dosage of IL10 could activate the cytotoxicity of intratumoral CD8+ T cells and induce the proliferation of these cells (20–23), contributing to a decrease in tumor burden (9) or tumor clearance (18, 19). A recent study has shown that pegylated IL10 (pegylodecakin, AM0010) induces tumor-specific CD8+ T-cell invigoration and polyclonal T-cell expansion in individuals with renal cell cancer (RCC; ref. 9). Furthermore, one study showed that the combination of AM0010 and 5-FU/LV and oxaliplatin (FOLFOX) was well tolerated in patients with metastatic pancreatic adenocarcinoma (PDAC; ref. 46). An increase in the levels of Th1 and Th2 cytokines was observed in the serum of patients receiving AM0010 in combination with FOLFOX. However, the levels of the immune suppressive molecule TGFβ3 and Th17 cytokines were decreased in the serum of patients treated with AM0010 in combination with FOLFOX, although the median progression-free survival (mPFS) was 3.5 months and the median overall survival (mOS) has not been reached but appears to be beyond 10.2 months (46). This regimen is currently being studied in a

Table 1. Relationship between the expression of Aurora-A and the clinicopathologic features of colorectal cancers.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n = 25)</th>
<th>Aurora-A high (n = 12)</th>
<th>Aurora-A low (n = 13)</th>
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<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
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<td>66.7</td>
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</table>
phase III trial (NCT02923921; 46). All of these observations indicated that inducing IL10 secretion could be a useful treatment strategy, although IL10 has immunosuppressive effects.

Overall, this study demonstrated that inhibition of Aurora-A activity by alisertib or deletion of the AURKA gene induced infiltration and expansion of intratumoral CD8+ T cells by enhancing IL10.

Disclosure of Potential Conflicts of Interest
K. Zheng reports a patent for a construction method of AURKA-CKO1-N conditional gene knockout mouse model (pending). J. Yang reports a patent for a construction method of AURKA-CKO1-N conditional gene knockout mouse model (pending). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
J. Han: Data curation, formal analysis, validation, investigation, methodology and writing-original draft. Z. Jiang: Writing-review and editing. X. Chen: Data curation and validation.

References

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