

Optimal dosing regimen of CD73 blockade improves tumor response to radiotherapy through iCOS downregulation

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ABSTRACT

Background Irradiation (IR) and immune checkpoint inhibitor (ICI) combination is a promising treatment modality. However, local and distance treatment failure and resistance can occur. To counteract this resistance, several studies propose CD73, an ectoenzyme, as a potential target to improve the antitumor efficiency of IR and ICI. Although CD73 targeting in combination with IR and ICI has shown attractive antitumor effects in preclinical models, the rationale for CD73 targeting based on CD73 tumor expression level deserves further investigations.

Methods Here we evaluated for the first time the efficacy of two administration regimens of CD73 neutralizing antibody (one dose vs four doses) in combination with IR according to the expression level of CD73 in two subcutaneous tumor models expressing different levels of CD73.

Results We showed that CD73 is weakly expressed by MC38 tumors even after IR, when compared with the TS/A model that highly expressed CD73. Treatment with four doses of anti-CD73 improved the TS/A tumor response to IR, while it was ineffective against the CD73 low-expressing MC38 tumors. Surprisingly, a single dose of anti-CD73 exerted a significant antitumor activity against MC38 tumors. On CD73 overexpression in MC38 cells, four doses of anti-CD73 were required to improve the efficacy of IR. Mechanistically, a correlation between a downregulation of iCOS expression in CD4⁺ T cells and an improved response to IR after anti-CD73 treatment was observed and iCOS targeting could restore an impaired benefit from anti-CD73 treatment.

Conclusions These data emphasize the importance of the dosing regimen for anti-CD73 treatment to improve tumor response to IR and identify iCOS as part of the underlying molecular mechanisms. Our data suggest that the selection of appropriate dosing regimen is required to optimize the therapeutic efficacy of immunotherapy–radiotherapy combinations.

BACKGROUND

Immune checkpoint inhibitor (ICI) therapies are considered the new standard of care for the treatment of several cancers¹ and the combination of irradiation (IR) and ICI has

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Even if the combination of irradiation and immunotherapy can ameliorate the efficacy of irradiation, some tumors can develop resistance resulting in treatment failure, hence we need to identify new biomarkers and novel approaches in order to optimize the effectiveness of the radio-immunotherapy combination.

WHAT THIS STUDY ADDS

⇒ Our data highlight for the first time the strong link between the expression level of CD73 and the optimal CD73 blockade dosing regimen to improve the tumor response to irradiation.

⇒ We identify iCOS signaling as a part of the underlying molecular mechanism. CD73 combined to PD-L1/PD-1 blockade synergize with radiation therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Overall, our data emphasize the importance of the appropriate dosing regimen and sequencing to optimize the therapeutic efficacy of immunotherapy–radiotherapy combinations underscoring the need for a personalized medicine approach to finely adjust dosing of checkpoint inhibitors for an optimal result in combination with radiotherapy.

been widely tested.^{2,3} Even if the combination of IR and ICI can ameliorate the efficacy of IR,^{4,5} some tumors can develop resistance resulting in treatment failure.^{6–9} To counteract this resistance to ICI and IR, several studies propose CD73 as a potential target to improve the antitumor efficiency of IR and ICI.^{10–12} CD73 is an ectoenzyme (ecto-50-nucleotidase, Ecto50NTase) expressed by cancer cells, immune cells, and the vasculature. By converting AMP into adenosine, CD73 plays a pivotal role in scavenging extracellular ATP to generate immunosuppressive adenosine in the microenvironment.¹³

Adenosine is a major inhibitor of effector T cell and natural killer cell antitumor functions, whereas extracellular ATP is an essential inhibitor of tumor cell proliferation and an important sensor molecule which attracts professional antigen-presenting cells to the tumor site.¹² CD73 is also directly involved in T-cell survival and function.^{14 15}

Preclinical studies showed promising antitumor effects for CD73 blockade in melanoma and prostate cancer cells,¹⁶ and anti-metastatic effects in several preclinical models.^{17 18} When combined with IR, CD73 blockade showed promising antitumor effects in breast cancer models.¹⁰ It is well established that the efficacy of anti-cancer therapy, especially immunotherapy, depends on the function and activation of T cells. Activation of conventional T cells begins following interaction of the T-cell receptor (TCR) with MHC (major histocompatibility complex) class I or class II-peptide complexes. Then, an important secondary co-stimulatory signal must be delivered in concert with TCR stimulation to allow proper T-cell activation.¹⁹ CD28 ligation had been shown to play a critical role in providing the required 'second signal' to promote cellular proliferation and survival following T-cell activation.^{20 21} The inducible T cell co-stimulator (iCOS, a new member of the immunoglobulin (Ig) family) has been identified as a new secondary co-stimulatory molecule.^{22 23} ICOS is not constitutively expressed on naive T cells, but is instead rapidly induced following TCR cross-linking and/or CD28 co-stimulation.²² ICOS co-stimulation, appears to play a complex role in dictating the profile of adaptive immune response by regulating Th1, Th2, and Th17 immunity.²⁴ The expression level of iCOS in CD4⁺ T lymphocytes has been reported to play a pivotal role in tumor tissue. Thus, a high expression level of iCOS was associated with tumor progression. By contrast a low expression level was associated with tumor control.²⁵ Furthermore, iCOS play an important role in both induction and immunosuppressive function of regulatory T cells (Tregs).²⁶

Despite the major advance in the field,^{27 28} the tumor response to immunotherapy remains heterogeneous, hence we need to identify new biomarkers and novel approaches in order to optimize the effectiveness of immunotherapy alone or combined with other anticancer treatments such as radiotherapy.

Here, we show the importance of the expression level of CD73 in the tumor response to CD73 blockade in combination with IR, and provide evidence of a strong link between the expression level of CD73 and the definition of the optimal dosing regimen for anti-CD73 to improve tumor response to IR. We identify iCOS signaling as a potential mechanism involved in the tumor response to CD73 blockade, in combination with IR. In MC38 tumors iCOS expression level decreased in CD4⁺ T lymphocytes after one dose of anti-CD73, and this was associated with an antitumor effect. However, iCOS expression level remained high in tumors treated with four doses of anti-CD73, which was associated with tumor progression.

Then, we reinforce the interest of blocking CD73 in combination with IR and ICI like anti-PD-L1.

METHODS

Cell lines

The MC38 tumor cell line was derived from C57BL/6 murine colon adenocarcinoma cells and was purchased from Kerastat. TS/A mouse mammary adenocarcinoma cells were purchased from Sigma-Aldrich. Cell lines were routinely screened for mycoplasma contaminations using the MycoAlert mycoplasma detection kit (Lonza).

Generation of CD73-overexpressing MC38 cells

CD73^{high} MC38 cells were obtained by transfection of MC38 cells with a plasmid encoding CD73 cDNA, the mus musculus 5' nucleotidase, ecto (Nt5e) cDNA delivered in pcDNA3.1⁺/C-(K)DYK vector (GenScript). Transfection was performed using the Lipofectamine 3000 (Thermo Fisher) following the manufacturer's protocol. 48 hours post-transfection, cells were treated with a selection antibiotic (G418, Sigma-Aldrich) for 15 days. Cells were then amplified in 0.6 mg/mL G418.

Animals

Animal procedures were performed according to protocols approved by the Ethical Committee CEEA 26 and in accordance with recommendations for the proper use and care of laboratory animals. For the subcutaneous tumor model, female BALB/c and C57BL/6 mice (8 weeks old) were purchased from Janvier Laboratories (France) and housed in the Gustave Roussy animal facility.

Subcutaneous tumor models

For tumor engraftment, 10⁶ MC38 or CD73^{high} MC38 cells in a 50 μ l volume (phosphate-buffered saline (PBS)) were injected subcutaneously in C57BL/6 mice. When tumors reached ~80–100 mm³, mice were randomly allocated to different treatment groups. 10⁵ TS/A cells in a 50 μ l volume (PBS) were injected subcutaneously in BALB/c mice. When tumors reached ~60–70 mm³, mice were randomly allocated to different treatment groups. Tumor size was measured with an electronic caliper. Tumor volume was estimated from two-dimensional tumor measurements (volume=length \times width²/2). The ethical endpoint for survival was a tumor volume exceeding 1200 mm³.

Irradiation

Subcutaneous tumors were locally irradiated using a Varian Tube NDI 226 (X-ray machine; 250 Kev, tube current: 15 mA, beam filter: 0.2 mm Cu). A single dose of 12 Gy was locally administered to the tumors at a dose rate of 1.08 Gy/min.

In vitro cell irradiation was performed using the XRAD320 machine (320kV, 4mA, 2mm AL Filter) in a single fraction of 6 and 12 Gy at a dose rate of 1.03 Gy/min.

Antibodies and in vivo treatments

The anti-CD73 (clone 2C5), the anti-PD-L1 (clone 80), the mouse IgG1 NIP228 isotype control and the mouse IgG1 D265A isotype control antibodies were supplied by MedImmune. Antibodies were administered intraperitoneally (i.p.) at 10 mg/kg at the indicated time points. For anti-CD73, two administration sequences were tested: (1) one dose, starting 1 day before IR and (2) four doses, starting 1 day before IR, and then twice a week for a total of four doses. Anti-PD-L1 was administered and began on the same day as IR, then twice a week for a total of four administrations. Anti-iCOS mAb (clone 7E.17G9) and the Rat IgG2b isotype control were purchased from Bio X Cell and i.p injected at 100 µg/mouse starting 2 days post-IR and then 6 and 9 days post-IR for a total of three injections.

Tumor dissociation

Tumors were digested using the Tumor Dissociation Kit (Miltenyi Biotec) for 40 min at 37°C and 1500 rpm. The cells from the tumors were filtered using cell strainers (70 µm, Miltenyi Biotec) and used for flow cytometry experiments.

Flow cytometry cell analysis

Purified anti-mouse CD16/32 (clone 93, BioLegend) was used for Fc receptor blocking.

For in vitro cell IR experiments, anti-CD73 (REA778, Miltenyi Biotec) was used at the appropriate dilutions.

For tumor-infiltrating immune cell staining, anti-CD45 (REA737), anti-CD4 (REA604), anti-CD8a (REA983), anti-NK1.1 (REA1162), anti-FoxP3 (REA788), anti-CD73 (REA778, Miltenyi Biotec), anti-CD19 (1D3, BD), anti-CD11b (M1/70) and anti-iCOS (C398.4A, BioLegend) antibodies were used. For membrane staining, cells were incubated with the antibody panel at the adapted concentrations for 20 min at 4°C. For FoxP3 staining, the Foxp3/Transcription Factor Staining Buffer Set (BD Biosciences) was used according to the manufacturer's instructions. Samples were washed in FACS buffer and resuspended in 200 µL FACS buffer before acquisition. Samples were acquired on an LSR Fortessa X20 (BD, Franklin Lakes, New Jersey) with FACSDiva software, and data were analyzed with FlowJo V.10.0.7 software (Tree Star, Ashland, Oregon, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism V.9 (GraphPad, California, USA). Survival data were analyzed using the Kaplan-Meier and log-rank (Mantel-Cox) tests for survival distribution. Two-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to detect differences in the tumor growth. One-way ANOVA followed by Tukey test was used to detect differences among multiple treatment groups for flow cytometry data. A p value equal to or less than 0.05 was considered significant (*p<0.05, **p<0.01,

p<0.001, *p<0.0001). Data are expressed as the mean±SEM.

RESULTS

Tuning of CD73 blockade is required to improve the response of low CD73-expressing MC38 tumors to IR

To investigate whether the colon MC38 tumors could be affected by the CD73 mAb treatment in combination with IR, we injected the MC38 tumor cells subcutaneously in C57BL/6 mice. Once tumors reached a mean size of 80–100 mm³, mice were treated with four doses of CD73 mAb in combination with IR as previously described for the breast cancer models¹⁰ (figure 1A). Four doses of CD73 mAb combined with IR had no effect on MC38 tumor growth, nor on survival rate when compared with IR alone (figure 1B and C). To investigate the mechanism underlying the lack of activity of the combined treatment, we analyzed the membrane expression of CD73 in both MC38 and TS/A cells. The basal level of CD73 membrane expression in MC38 tumor cells was lower when compared with TS/A tumor cells and was slightly, even if significantly, increased by a 12 Gy IR (figure 1D). Given the lower expression of CD73 in MC38 cells, we adapted the dosing regimen of CD73 mAb treatment according to the expression level of CD73 in MC38 cells and animals were treated with only one dose of CD73 mAb, rather than four. Interestingly, one dose of CD73 mAb combined with IR induced a delay in tumor growth, unlike four doses of CD73 mAb (figure 1B). In addition, one dose of CD73 mAb combined with IR improved survival rate when compared with other treatment groups (figure 1C). Conversely, in TS/A tumors, which expressed high levels of CD73, four doses of CD73 antibody were required to improve antitumor effects of IR (figure 1F and G) while a single dose was ineffective.

Altogether, our results demonstrated that CD73 blockade regimen affects the tumor response to IR suggesting that CD73 expression level may dictate the response of tumor models to CD73 blockade treatment.

CD73 expression level controls the MC38 tumor response to CD73 blockade treatment

To confirm the impact of CD73 expression level in the MC38 tumor response to CD73 blockade combined with IR, we generated MC38 cells stably overexpressing CD73 (CD73^{high} MC38) (figure 2A). IR significantly increased the level of CD73 membrane expression in CD73^{high} MC38 cells compared with MC38 control (figure 2B).

Four doses of CD73 antibody combined with IR significantly delayed tumor growth and improved survival in comparison to IR alone in mice bearing CD73^{high} MC38 tumors (figure 2D and E), while one dose of CD73 antibody combined with IR significantly delayed tumor growth and improved survival in comparison to IR alone in mice bearing MC38 control tumors (figure 2F and G).

All these data demonstrate that the expression level of CD73 on MC38 tumor cells modifies the tumor response to CD73 blockade, in combination with IR.

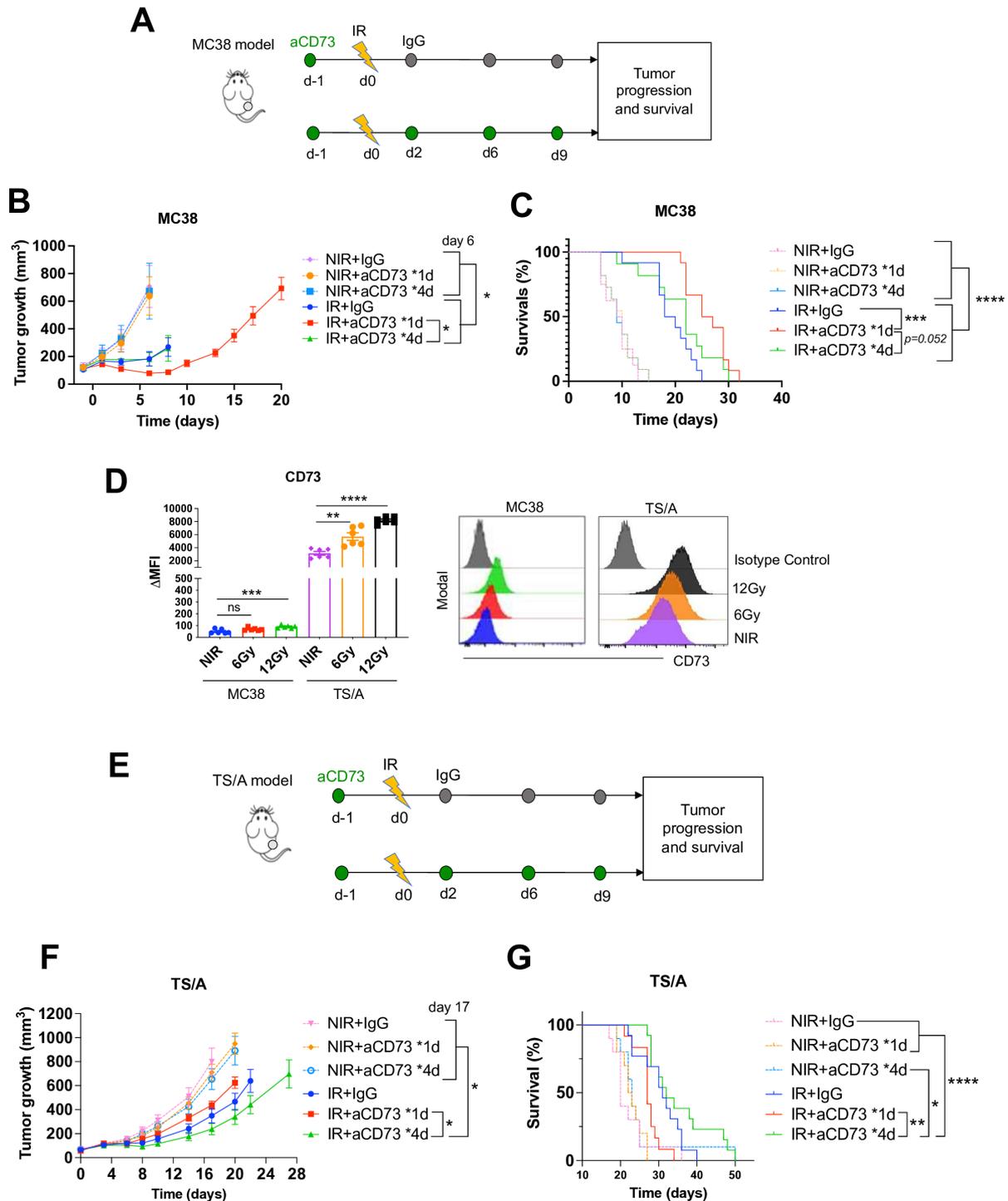


Figure 1 Tuning of CD73 blockade is required to improve the response of low CD73-expressing MC38 tumors to IR. (A) MC38 tumor cells were injected subcutaneously in C57BL/6 mice and when tumors reached 80–100 mm³, tumor were irradiated at 12 Gy, and mice were treated with anti-CD73 commencing 1 day before IR then 2, 6 and 9 day post-IR. (B) Tumor growth was monitored in treated mice. (C) The Kaplan-Meier survival curves for the treated mice are shown. Data were obtained from two independent experiments and are represented as the mean±SEM. n=11–12, *p<0.05 ****p<0.0001 (two-way ANOVA). (D, right panel) Representative histograms of CD73 expression in MC38 and TS/A cells 24-hour post-IR at 6 and 12 Gy compared with non-irradiated (NIR) cells. (D, left panel) cultured cells were analyzed by flow cytometry for their CD73 expression, which is represented as the mean fluorescence intensity ($\Delta\text{MFI} = \text{MFI of the isotype control} - \text{MFI of stained cells}$). Data were obtained from two independent experiments and are represented as the mean±SEM. n=6, **p<0.01, ***p<0.001, ****p<0.0001 (two-way ANOVA). (E) TS/A tumor cells were injected subcutaneously in BALB/c mice and when tumors reached 60–70 mm³, tumor were irradiated at 12 Gy, and mice were treated with anti-CD73 commencing 1 day before IR then 2, 6 and 9 days post-IR. (F) Tumor growth was monitored in treated mice. (G) The Kaplan-Meier survival curves for the treated mice are shown. (I) Tumor growth is shown for individual mice in each treatment group. Data were obtained from two independent experiments and are represented as the mean±SEM. n=12–13, **p<0.01 (two-way ANOVA). ANOVA, analysis of variance; IgG, immunoglobulin G; IR, irradiation.

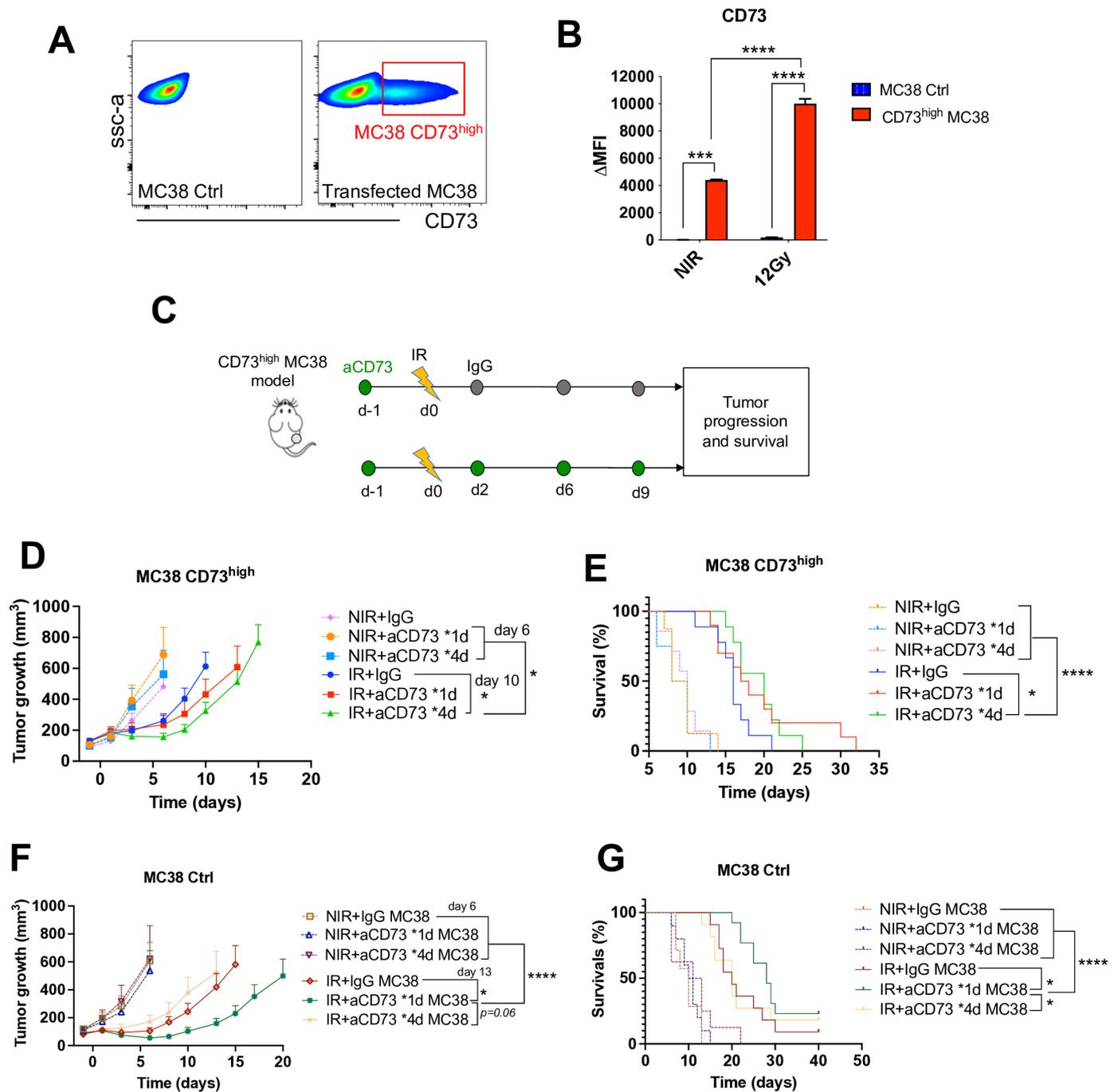


Figure 2 CD73 expression level controls the MC38 tumor response to CD73 blockade treatment. (A) Representative histograms of transfected and non-transfected MC38 cell with CD73 gene analyzed by flow cytometry. (B) Cultured MC38 control (Ctrl) and CD73^{high} MC38 cells were analyzed by flow cytometry 24-hour post-IR at 12 Gy for their CD73 membrane expression, which is represented as the mean fluorescence intensity ($\Delta\text{MFI} = \text{MFI of the isotype control} - \text{MFI of stained cells}$). Data were obtained from two independent experiments and are represented as the mean \pm SEM. $n=6$, $***p < 0.001$, $****p < 0.0001$ (one-way ANOVA). (C) CD73^{high} MC38 tumor cells were injected subcutaneously in C57BL/6 mice and when tumors reached 80–100 mm³, tumor were irradiated at 12 Gy, and mice were treated with anti-CD73 starting 1 day before IR then 2, 6 and 9 days post-IR. (D, F) Tumor growth was monitored in treated mice. (E, G) The Kaplan-Meier survival curves for the treated mice are shown. Data were obtained from two independent experiments and are represented as the mean \pm SEM. $n=13-14$, $*p < 0.05$, $***p < 0.001$ (two-way ANOVA). ANOVA, analysis of variance; IgG, immunoglobulin G; IR, irradiation; NIR, non-irradiated; ssc-a, side scatter-a.

CD73 blockade treatment regimen affects the expression level of iCOS in tumor infiltrating CD4⁺ T lymphocytes

To assess the molecular profile of MC38 tumors following the two CD73 blockade regimens, we performed

targeted ultra high performance liquid chromatography (UHPLC)/mass spectrometry analyses and demonstrated that the ratio of adenosin/AMP, ADP/AMP and ATP/AMP significantly decreased after either one dose, or

four doses of anti-CD73 (online supplemental figure 1) confirming biological activity of anti-CD73.

To characterize the tumor-infiltrating immune cell populations following the two CD73 blockade regimens, we performed flow cytometry analyses. The total number of both myeloid and lymphoid cells were not affected (online supplemental figure 2). After gating on CD4⁺ T lymphocytes (online supplemental figure 3) the membrane expression of iCOS in CD4⁺ T lymphocytes in MC38 tumors decreased after one dose of anti-CD73 however, they were not affected by four doses of anti-CD73 (figure 3A). Furthermore, the per cent of iCOS⁺ CD4⁺ T lymphocytes decreased only in the tumors treated with one dose of anti-CD73 combined to IR (figure 3B). The per cent of Treg trended to decrease in MC38 tumors treated with one dose of anti-CD73 combined to IR (online supplemental figure 4A). By contrast, in MC38 tumors overexpressing CD73 we observed a downward trend in the expression of iCOS in CD4⁺ T lymphocytes after four doses of anti-CD73, and no modulation after a single dose of anti-CD73 (figure 3C). In addition, the per cent of iCOS⁺ CD4⁺ T lymphocytes tended to decrease only in the CD73^{high} MC38 tumors treated with four doses of anti-CD73 combined with IR (figure 3D).

Subsequently, we assessed iCOS expression level in CD4⁺ T lymphocytes in TS/A tumor models. The membrane expression level of iCOS in CD4⁺ T lymphocytes decreased after four doses of anti-CD73 but was not affected by one dose of anti-CD73 (figure 3E). In the same way, the per cent of iCOS⁺ CD4⁺ T lymphocytes significantly decreased in the tumors treated with four doses of anti-CD73 combined with IR (figure 3F) and the per cent of Treg trends to decrease in TS/A tumors treated with four doses of anti-CD73 combined with IR (online supplemental figure 4B).

Altogether, our data demonstrated that iCOS expression level in CD4⁺ T lymphocytes correlates with the efficacy of CD73 blockade in combination with IR.

iCOS signaling is involved in CD73 blockade-mediated antitumor effect in MC38 tumor model

To investigate the role of iCOS in the antitumor effect mediated by CD73 blockade in combination with IR, mice bearing MC38 tumors were treated with CD73 antibody in combination with IR and iCOS mAb (figure 4A). As expected, four doses of anti-CD73 combined with IR had no antitumor effect (figure 4B and F). Interestingly, iCOS blockade improved the antitumor effect in the group treated with four doses of anti-CD73 and IR (figure 4B and F). Furthermore, iCOS blockade improved the survival rate in the group treated with four doses of anti-CD73 and IR when compared with other treatment groups (figure 4C). Conversely, iCOS blockade did improve neither the antitumor effect, nor the survival rate in the groups treated with one dose of anti-CD73 and IR (figure 4D–4F).

Our data suggests that iCOS signaling in CD4⁺ T lymphocytes could be an important mechanism involved in CD73

blockade-mediated antitumor effect, and propose iCOS as a potential target to improve antitumor effects of anti-CD73 treatment and IR.

One dose of anti-CD73 improves the antitumor effect of anti-PD-L1 and IR treatment in MC38 tumor model

Subcutaneous MC38 tumor model is known to be a well-responder to ICI alone or in combination with IR.^{29 30} In order to assess whether one dose of anti-CD73 could improve this antitumor effect, we injected the MC38 tumor cells subcutaneously in C57BL/6 mice. Once tumors reached a mean size of 80–100 mm³, mice were treated with one dose of CD73 antibody combined with IR and PD-L1 mAb (figure 5A). Interestingly, one dose of anti-CD73 significantly improved the antitumor effect of IR and anti-PD-L1 combination (figure 5B and D) and improved survival (figure 5C).

Altogether, our data demonstrates that in the CD73 low expressing MC38 tumor model, one dose of anti-CD73 alone is sufficient to increase the antitumor response to anti-PD-L1 and IR combination.

DISCUSSION

In the clinic, the expression of the immune-checkpoint target (eg, PD-L1) in tumor cells is a critical predictor of response to ICI. However, no biomarkers are used to adapt the treatment schedule according to the level expression of the target. Most preclinical studies are designed according to the positive expression of the immune-checkpoint target in selected tumor cells and overall, in preclinical studies ICI were usually given every 2–3 days for at least 2 weeks.^{3 4 10} Here we describe a new insight for the importance of a well-chosen CD73 inhibitor dosing regimen according to the expression level of CD73. The following conclusions were reached: (1) in MC38 tumor model harboring low expression level of CD73 one dose of anti-CD73 improved the antitumor effect of IR, by contrast, four doses of anti-CD73 combined to IR had no effect; (2) the improvement of IR antitumor efficacy in MC38 model by one dose of anti-CD73 was associated with a low expression of iCOS in T CD4⁺ lymphocytes and a trend towards a decrease in Treg per cent. Also, the lack of the antitumor effect of four doses of anti-CD73 combined with IR in treated MC38 tumors was associated with a high level of iCOS surface expression in T CD4⁺ lymphocytes; (3) iCOS depletion did not improve the antitumor effect of one dose of anti-CD73 plus IR and when MC38 tumors were treated with four doses of anti-CD73 plus IR, iCOS depletion improved the antitumor efficiency of the combined treatment; (4) one dose of anti-CD73 significantly improved the antitumor efficacy of IR and anti-PD-L1 combination in the MC38 tumor model.

Several studies identified CD73 as a potential target to improve the antitumor effect of IR and/or ICI since the expression level of CD73 was found to be high in tumor cells.^{10 11} The proliferation of T cells obtained

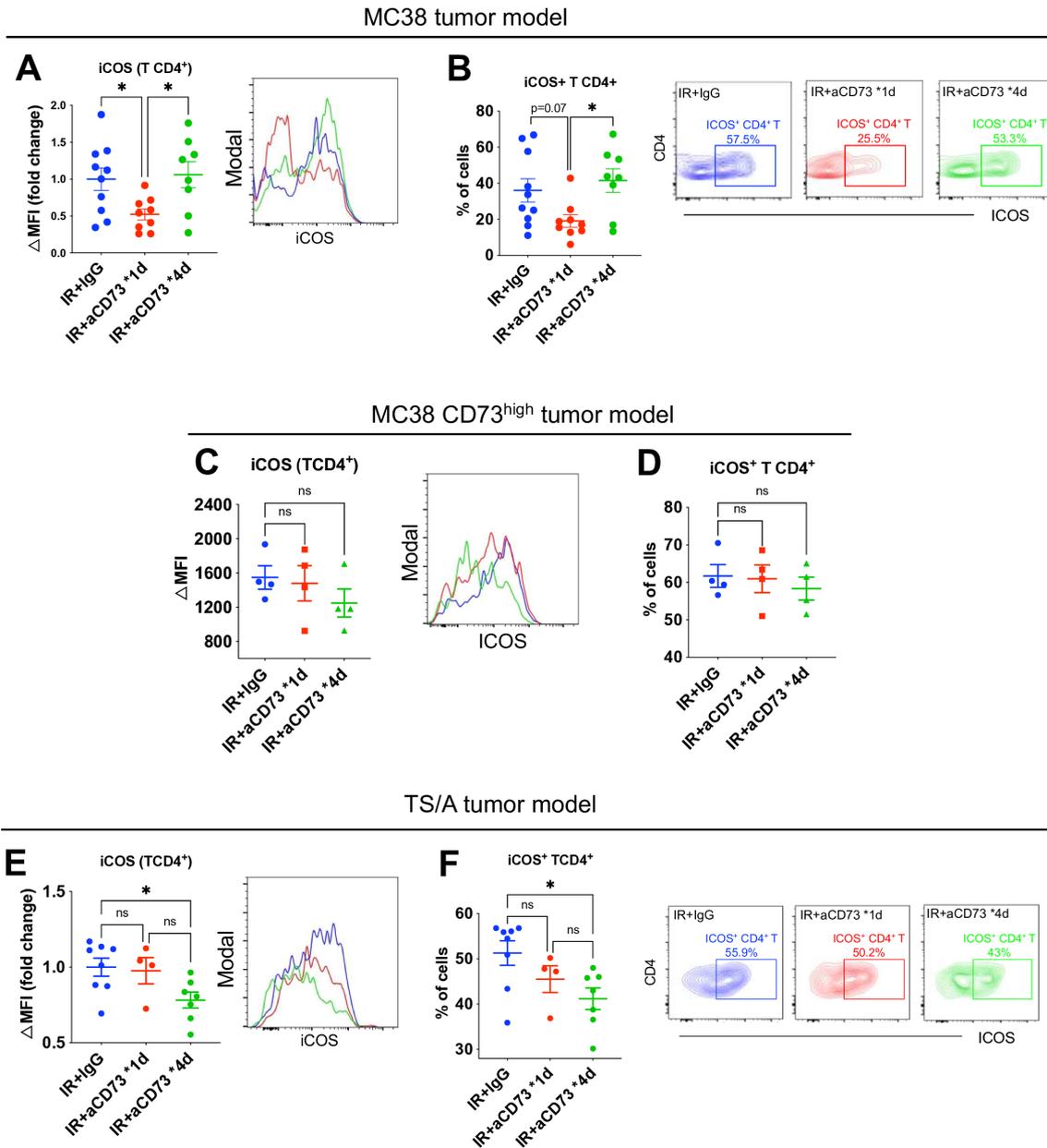


Figure 3 CD73 blockade treatment regimen affects the expression level of iCOS in tumor infiltrating CD4⁺ T lymphocytes. C57BL/6 mice with the subcutaneous MC38 tumors and BALB/c mice with subcutaneous TS/A tumors were irradiated and treated with either one dose or four doses of anti-CD73 starting 1 day before IR, then 2, 6 and 9 days post-IR. At day 10 post-IR, tumors were harvested and analyzed for immune infiltrating tumor cells by flow cytometry. CD4⁺ T lymphocytes infiltrating MC38 tumor were analyzed by flow cytometry for iCOS (A, left panel) membrane expression, which is represented as the mean fluorescence intensity (Δ MFI=MFI of the isotype control - MFI of stained cells). (A, right panel) Representative histograms of iCOS expression, in CD4⁺ T lymphocytes infiltrating MC38 tumors. (B, left panel) The percentages of iCOS⁺ CD4⁺ T lymphocytes infiltrating MC38 tumor are presented for each treatment group. (B, right panel) Representative histograms of iCOS⁺ CD4⁺ T lymphocytes infiltrating MC38 tumor are presented for each treatment group. Data were obtained from two independent experiments and are represented as the mean \pm SEM. $n=8-10$, * $p<0.05$ (two-way ANOVA). CD4⁺ T lymphocytes infiltrating MC38 CD73^{high} tumors were analyzed by flow cytometry for iCOS (C, left panel) membrane expression, which is represented as the mean fluorescence intensity (Δ MFI=MFI of the isotype control - MFI of stained cells). (C, right panel) Representative histograms of iCOS expression, in CD4⁺ T lymphocytes infiltrating MC38 CD73^{high} tumors. (D) The percentages of iCOS⁺ CD4⁺ T lymphocytes infiltrating MC38 CD73^{high} tumors are presented for each treatment group. CD4⁺ T lymphocytes infiltrating TS/A tumor were analyzed by flow cytometry for their iCOS (E, left panel) membrane expression, which is represented as the mean fluorescence intensity (Δ MFI=MFI of the isotype control - MFI of stained cells). (E, right panel) Representative histograms of iCOS expression in CD4⁺ T lymphocytes infiltrating TS/A tumor. (F, left panel) The percentages of iCOS⁺ CD4⁺ T lymphocytes infiltrating TS/A tumor are presented for each treatment group. (F, right panel) Representative histograms of iCOS⁺ CD4⁺ T lymphocytes infiltrating MC38 tumor are presented for each treatment group. Data were obtained from two independent experiments and are represented as the mean \pm SEM. $n=4-8$, * $p<0.05$ (two-way ANOVA). ANOVA, analysis of variance; IgG, immunoglobulin G; IR, irradiation.

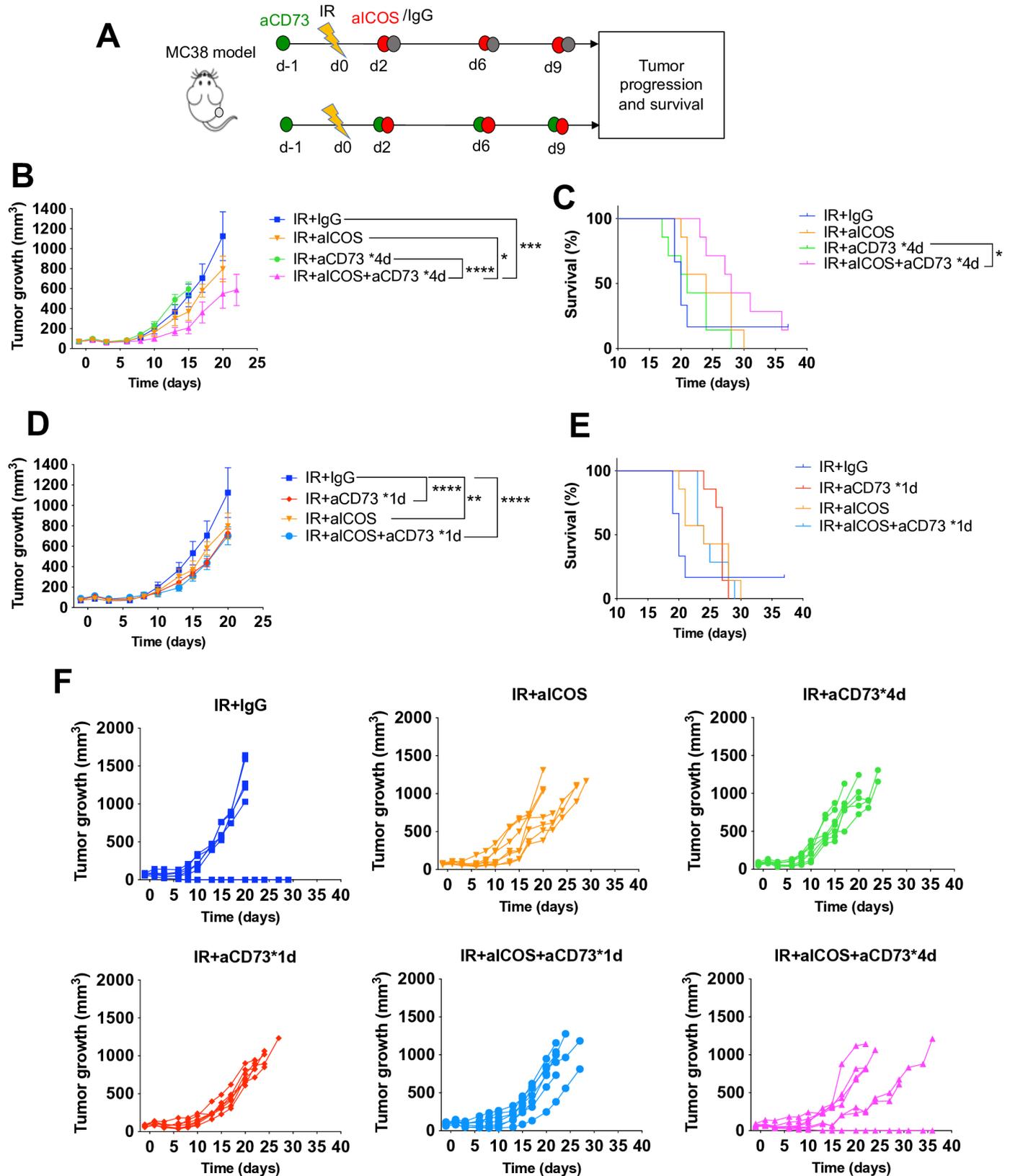


Figure 4 iCOS signaling is involved in CD73 blockade-mediated antitumor effect in MC38 tumor model. (A) MC38 tumor cells were injected subcutaneously in C57BL/6 mice and when tumors reached 80–100 mm^3 , tumor were irradiated at 12 Gy, and mice were treated with anti-CD73 (starting 1 day before IR then 2, 6 and 9 days post-IR) and anti-iCOS (starting 2 days post-IR, then 6 and 9 days post-IR). (B and D) Tumor growth was monitored in treated mice. (C and E) The Kaplan-Meier survival curves for the treated mice are shown. (F) Tumor growth is shown for individual mice in each treatment group. Data were obtained from two independent experiments and are represented as the mean \pm SEM. $n=6-7$, * $p<0.05$, **** $p<0.0001$, *** $p<0.001$ (two-way ANOVA). ANOVA, analysis of variance; IgG, immunoglobulin G; IR, irradiation.

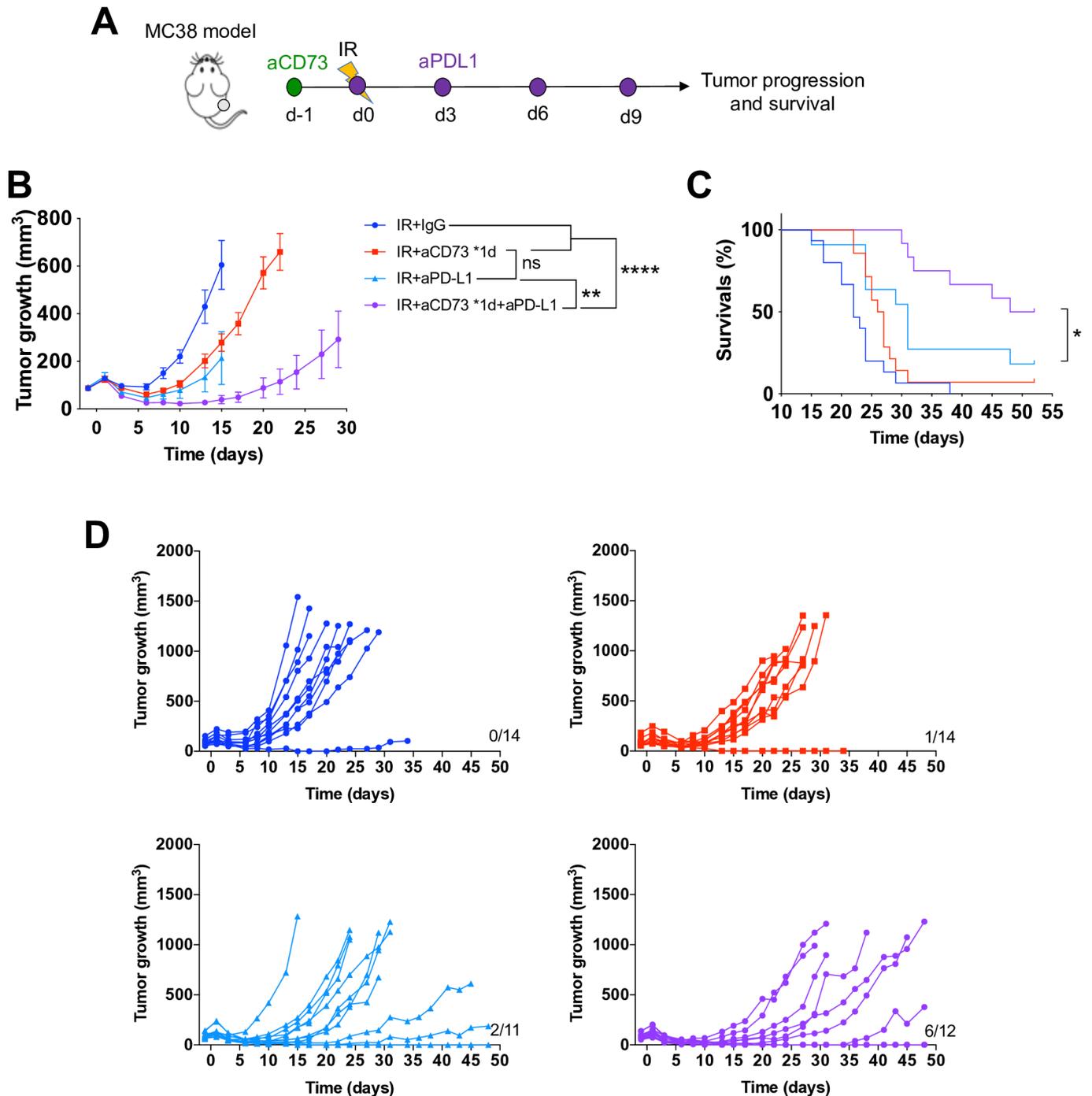


Figure 5 One dose of aCD73 improves the antitumor effect of aPD-L1 and IR treatment in MC38 tumor model. (A) MC38 tumor cells were injected subcutaneously in C57BL/6 mice and when tumors reached 80–100 mm³, tumor were irradiated at 12 Gy, and mice were treated with one dose of anti-CD73 (starting 1 day before IR) and anti-PD-L1 (starting the same day as IR, then 3, 6 and 9 days post-IR). (B) Tumor growth was monitored in treated mice. (C) The Kaplan-Meier survival curves for the treated mice are shown. (D) Tumor growth is shown for individual mice in each treatment group. Data were obtained from two independent experiments and are represented as the mean±SEM. n=11–14, *p<0.05, **p<0.01, ****p<0.0001 (two-way ANOVA). ANOVA, analysis of variance; IgG, immunoglobulin G; IR, irradiation.

from healthy donors and patients with breast cancer was restored by CD73 blockade antibody.¹²

Our data showed that MC38 tumor model expressed very low levels of CD73 and its induction by IR was not important, in contrast to what is observed in mouse breast cancer cells.¹⁰ However, MC38 tumor microenvironment is composed of immune cells³¹ that strongly

express CD73.¹¹ All immune cells using CD73 signaling for their active metabolism work in a complex network to impact the tumor response to treatments^{12 32 33} giving the rationale for testing CD73 blockade in MC38 model. Accordingly, we tested two administration sequences of CD73 antibody in combination with IR in MC38 subcutaneous tumor model: (1) four doses as recommended

by the supplier and according to the work from De Maria Group¹⁰ and (2) one dose since the MC38 cells do not express high level of the target and we hypothesized that this model does not require high amount of CD73 antibody. Interestingly, our data demonstrated that in MC38 tumor model one dose of anti-CD73 improved the anti-tumor effect of IR, by contrast, four doses of anti-CD73 combined to IR has no effect. In TS/A tumor model harboring high CD73 expression level four doses of anti-CD73 are required to improve the anti-tumor effect of IR suggesting that the expression level of CD73 in tumor cells dictates the response to anti-CD73 in combination to IR. Accordingly, in CD73^{high} MC38 tumor model, four doses of anti-CD73 improve the antitumor effect of IR. All our data suggest that the high occupancy/saturation of CD73 binding sites by anti-CD73 is detrimental. The link between the binding site occupancy and the efficacy of a specific dosing regimen for an agonist agent has already been described.³⁴ OX40 receptor occupancy by CD40 agonist between 20% and 40% was sufficient to yield antitumor activity and was associated with maximal enhancement of T-cell effector function by anti-OX40 treatment, whereas a receptor occupancy >40% led to a profound loss in OX40 receptor expression, and T-cell activity plateaued or diminished.³⁴

Our data demonstrated that anti-CD73 regimen affects iCOS expression in tumor-infiltrating T CD4⁺ cells. Interestingly, in MC38 tumor model, the improvement of IR antitumor efficacy by one dose of anti-CD73 was associated with a low expression of iCOS in T CD4⁺ lymphocytes and a trend towards a decrease in Treg per cent. The lack of the antitumor effect of four doses of anti-CD73 combined with IR in treated MC38 tumors was associated with a high level of iCOS surface expression in T CD4⁺ lymphocytes. The cell surface expression level of iCOS played a pivotal role in effector T-cell activity.^{24 25 35} ICOS^{low} T cells were associated with cytokine interleukin (IL)-2 and interferon (IFN)- γ and antitumor activity, whereas ICOS^{high} T cells were linked to anti-inflammatory cytokine IL-10.²⁵ Accordingly, our data showed that in MC38 tumor model iCOS depletion did not improve the antitumor effect of one dose of anti-CD73 plus IR and this could be explained by the low expression level of iCOS in this condition. By contrast, when MC38 tumors were treated with four doses of anti-CD73 plus IR, iCOS depletion improved the antitumor efficiency of combined treatments and this could be due to the increase of expression level of iCOS in this condition. Thus, our data suggest that a short sequence (one dose) of CD73 antibody combined with IR restored the antitumor activity of CD4⁺ T lymphocytes in MC38 tumor model via iCOS signaling. In non-small cell lung cancer, Park *et al* reported an inverse association between CD73 expression and tumor infiltration by activated CD4⁺ T cells. Furthermore, iCOS expression was positively correlated with CD73 expression.³⁶ Interestingly, it is well established that CD4⁺ T cells from naive mice that express high levels of iCOS produce a great amount of IL-10,²⁵ a cytokine frequently produced by Tregs. ICOS has

been reported to be directly linked with Treg induction. During bacterial infection, infected *Icos*^{-/-} mice exhibited delayed expansion and decreased total number of CD4⁺Foxp3⁺ Tregs when compared with wild-type (WT) mice. Most importantly, the number of CD4⁺CD25⁺Foxp3⁺Tregs in the lung of WT mice increased 1.8-fold after respiratory tolerance induction with 77% of lung-derived Tregs expressing ICOS.²⁶ Accordingly, our data showed that a low expression of iCOS in T CD4⁺ lymphocytes is associated with a trend towards a decrease in Treg per cent.

A study from the De Maria Group showed that CD73 blockade in combination with IR in the TS/A tumor model enhanced tumor infiltration by conventional type 1 dendritic cells (cDC1) in a type I IFN independent manner and enhanced the induction of systemic antitumor T-cell response.¹⁰ In our study, we observed that the antitumor efficacy of anti-CD73 and IR combination affected iCOS expression level in CD4⁺ T lymphocytes. This could be one of the molecular mechanisms involved in the antitumor efficacy of CD73 blockade combined with IR.

CD73 blockade in combination with IR and/or other ICI like CTLA-4 and PD-1 blockade had already been addressed and showed promising antitumor efficacy.^{10-12 37} Accordingly, our results indicate that one dose of anti-CD73 significantly improved the antitumor efficacy of IR and anti-PD-L1 combination in MC38 tumor model reinforcing the interest of blocking CD73 in combination with IR and ICI.³⁸ Currently, three clinical studies evaluating the anti-CD73 blocking effect alone or in combination with irradiation and/or immunotherapy are being recruiting (NCT03875573, RECF-004731, PACIFIC-9 (D9078C00001)) and all of them are designed to use at least four administrations of anti-CD73 antibody. It would be important to evaluate the expression level of CD73 in the tumors to adapt the administration schedule of CD73 blocking treatment to increase treatment efficiency and prevent any toxicities.

CONCLUSION

In summary, our data highlight the strong link between the expression level of CD73 and the optimal CD73 blockade dosing regimen to improve the tumor response to IR. The alteration of iCOS signaling could be part of the molecular mechanism involved in the improved response. Our findings provide a rationale for testing CD73 blockade in combination with IR in cancers expressing low levels of CD73 but with an adjusted dosing regimen. The concept of 'optimal dosing regimen' could be applied to other ICI and could be beneficial for several human cancers which lack the immune target expression and maybe it would pave the way for 'personalized immunotherapy treatments'.

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