Inhibition of invariant chain expression in dendritic cells presenting endogenous antigens stimulates CD4+ T cell responses and tumor immunity

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ABSTRACT

Induction of potent and sustained antiviral or antitumor immunity is dependent on the efficient activation of CD8\(^+\) and CD4\(^+\) T cells. While dendritic cells constitute a powerful platform for stimulating cellular immunity, presentation of endogenous antigens by dendritic cells transfected with nucleic acid-encoded antigens favors the stimulation of CD8\(^+\) T cells over that of CD4\(^+\) T cells. A short incubation of mRNA transfected dendritic cells with antisense oligonucleotides directed against the invariant chain enhances the presentation of mRNA encoded class II epitopes and activation of CD4\(^+\) T cell responses in vitro and in vivo. Immunization of mice with the antisense oligonucleotide treated dendritic cells stimulates a more potent and longer lasting CD8\(^+\) CTL response and enhances the antitumor efficacy of dendritic cell-based tumor vaccination protocols. Transient inhibition of invariant chain expression represents a simple and general method to enhance the stimulation of CD4\(^+\) T cell responses from endogenous antigens.
INTRODUCTION

Immunization using DC loaded with tumor antigens is a potentially powerful method of inducing antitumor immunity\(^1\). An effective way of loading DC with tumor antigen is to transduce the DC with recombinant viral vectors or to transfet with mRNA encoding tumor antigens\(^2\). CD8\(^+\) cytotoxic T cells (CTL) are an important effector arm in the antitumor immune response and the induction of potent CTL responses has been the main goal in developing immunotherapeutic strategies for cancer\(^3,4\). Accumulating evidence, however, suggest that the CD4\(^+\) T cell response also plays a key role in tumor immunity\(^5,6\). CD4\(^+\) T cells provide important functions for the induction, expansion and persistence of CD8\(^+\) CTL\(^7\). Secretion of effector cytokines such as IFN\(\gamma\) by CD4\(^+\) T cells sensitizes tumor cells to CTL lysis via upregulation of MHC class I molecules, stimulates the innate arm of the immune system at the tumor site and, as was recently suggested, inhibits local angiogenesis\(^8\). The importance of the CD4\(^+\) T cell response in tumor immunity was highlighted in murine studies showing that CD4\(^+\) T cells can eradicate tumor in the absence of CD8\(^+\) T cells\(^9\text{--}11\) or constitute the dominant effector arm in the antitumor response\(^12\). Therefore, an optimal antitumor immune response will require the concomitant activation of both CD4\(^+\) and CD8\(^+\) T cells.

Endogenously expressed antigens, such as antigens expressed in DC transfected with mRNA, will be channeled preferentially into the class I processing pathway to activate the CD8\(^+\) T cell arm of the immune response\(^13\). Antigens of cytoplasmic origin which access the endocytic/lysosomal compartments can generate peptides for loading class II molecules and stimulate, albeit weak, CD4\(^+\) T cell responses\(^14\). Wu et al have shown that it is possible to further enhance the class II presentation of endogenous antigens by appending a leader sequence to the amino end and a lysosomal sorting signal to the carboxyl end of the endogenously expressed...
antigen\textsuperscript{15}. Engineering leader sequences and/or lysosomal/endosomal targeting signals is, however, not applicable to immunization with tumor derived antigenic mixtures such as tumor-derived mRNA. Furthermore, binding of endogenously derived peptides with nascent MHC class II molecules is inhibited by the invariant chain (see below).

MHC class II negative tumor cells transfected with MHC class II cDNA expression plasmids exhibit enhanced antitumor immunogenicity in mice\textsuperscript{16}. Co-expression of the invariant chain, however, abrogates the immunogenicity of the class II transfected tumor cells\textsuperscript{17}. This observation, and the findings that presentation of endogenous peptides is often, but not always, favored in cells expressing class II molecules in the absence of invariant chain\textsuperscript{16,18-20}, is illustrative of the natural role of invariant chain to prevent the association of endogenously derived class II-restricted peptides in the endosome or Golgi compartments with the nascent class II molecules\textsuperscript{21,22}. Transfection of tumor cells with patient-specific class II alleles is not a practical approach to stimulate antitumor CD4\textsuperscript{+} T cell immunity in cancer patients. Recently, Humphreys and colleagues have described a strategy whereby class II as well as invariant chain expression is induced in tumor cells, either by transfection with a CIITA expression plasmid or by treatment with IFN\textgamma, followed by selective downregulation of invariant chain expression using antisense oligonucleotides directed against invariant chain (Ii AS ODN)\textsuperscript{23}. This approach is, however, limited by tumor tissue availability, transfectability of primary human tumors or their responsiveness to IFN\textgamma mediated induction of class II expression.

In this study we tested the hypothesis that antisense ODN-mediated inhibition of invariant chain expression in mRNA transfected DC will lead to enhanced presentation of class II-restricted epitopes, enhanced induction of CD4\textsuperscript{+} T cell and CD8\textsuperscript{+} CTL responses in mice, and improved tumor immunity. Since vaccination with tumor mRNA-transfected DC does not require the identification of the effective tumor antigens in each cancer patient and is not limited by tumor
tissue availability, this approach could represent a broadly useful method to augment antitumor CD4+ T cell immunity, alongside CD8+ T cell immunity.

METHODS

Mice, cell lines and reagents.

6-8 weeks old C57BL/6 mice (H-2b) were obtained from the Jackson Laboratory, Bar Harbor, ME. Animal studies were approved by the Duke Institutional Animal Care & Use Committee (IACUC). Cell lines used were B16/F10.9 (C57BL/6, H-2b) melanoma tumor cells24, B16/F10.9-OVA, chicken ovalalbumin (OVA) cDNA transfected B16/F10.9 cells25, and RMA-S cells (C57BL/6, H-2b). OVA-specific class I and class II restricted RF33.70 and MF2.2D9 T-cell hybridomas, respectively, were a kind gift from Dr. Kenneth Rock.

Peptides and oligonucleotides (ODN).

OVA peptide (H-2Kb restricted, SIINFEKL, aa 257-264)26, VSV peptide (H-2Kb restricted, RGYVYQGL)27, OVA peptide (I-Ab restricted, IINFEKLTEWTSSNVMEER, aa 258-276)28 and VSV II peptide (I-Ab-restricted, SSKAQVFEHPHIQDAASQL)27 were purchased from Research Genetics (Huntsville, AL). The following phosphorothioate-modified ODNs were synthesized: AE40 (5’-TTGGTCATCCATGGCTCT-3’) and AE54 (5’-TGGTCATCCATGGCTCTA-3’), correspond to previously described invariant chain (Ii)antisense ODNs23. SE40 (5’-TCTCGG TACCTACTGGTT-3’), a scrambled sequence of AE40, SE46 (5’-ATGGATGACCAACGCGAC-3’) and SE54 (5’-TAGAGCCATGGATGACCA-3’), the complementary (sense) strand of AE40 and AE54, respectively.
RNA preparation

Cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). mRNA was isolated from total RNA using MACS mRNA isolation kit (Miltenyi Biotec, Auburn, CA). The plasmids, pGEM4Z/OVA/A64, pGEM4Z/GFP/A64 and pGEM4Z/Flu/A64, used for in vitro transcription of OVA, GFP and influenza (Flu, M1) mRNA respectively, were previously described. Murine tyrosinase-related protein 2 (TRP-2) mRNA and a truncated form of OVA from which the first 40 aa were deleted were produced by in vitro transcription from a cDNA fragment amplified by RT-PCR.

In vivo depletion of CD4+ or CD8+ T cells

150 µg anti-CD4+ ascites (GK1.5) or 200 µg anti-CD8+ (53-6.72) was injected into mice i.p. at days –3, 0, +3 and +6 of tumor challenge. Greater than 98% and 94% of CD4+ or CD8+ T cells were specifically depleted under those conditions, respectively. Non-depleted control mice received injection of 200 µg mouse IgG.

Tumor challenge

Mice were immunized twice weekly i.p. with 2–4x10^5 electroporated DC in 200 µl PBS. After 10 days, the mice were challenged subcutaneously with 1x10^5 tumor cells in 200 µl PBS. For the treatment of pre-existing tumor, 3 x 10^4 B16/F10.9-OVA cells were injected subcutaneously at the right flank of C57BL/6 mice. The mice were then immunized i.p. with 5 x 10^5 RNA electroporated DC at 3, 7 and 14 days respectively after tumor inoculation. Site of tumor implantation was monitored daily for the appearance of palpable tumors. Tumor volume (smallest diameter^2 x largest diameter) was measured every other day starting at day 13-15 after tumor challenge. Mice were sacrificed when the diameter of the tumor reached 2 cm.
RESULTS

We used two phosphorothioate-modified antisense oligonucleotides (ODNs) described by Qiu et al.\textsuperscript{23} to test whether invariant chain expression can be inhibited in murine DC. Surface expression of invariant chain (CD74) is significantly reduced in DC treated with antisense (Ii AS), but not control, ODNs, whereas a partial inhibition was seen when both intracellular and cell surface expression were measured (Fig. 1A). Similar effects were seen with a second antisense and control ODN (Materials and Methods, data not shown). The specificity of the invariant chain antisense ODNs is demonstrated in Fig. 1B. No inhibition of CD40, CD80, CD86 or MHC class II I-A\textsuperscript{b} expression was seen in DC incubated with Ii AS ODNs. Invariant chain controls the proper folding and trafficking of the nascent MHC class II molecules and their appearance at the cell surface\textsuperscript{21,22}. However, this function is cell type dependent, since in many instances class II molecules can traffic to the cell surface in the complete absence of invariant chain\textsuperscript{30-33}. Consistent with our observations, invariant chain independent class II expression on the cell surface was seen in DC and activated macrophages, but not in B cells\textsuperscript{34,35}.

Figure 1
The presentation of MHC class I and class II chicken ovalbumin (OVA) epitopes is shown in Fig. 2. C75BL/6 (H-2\textsuperscript{b}) mice present an H-2K\textsuperscript{b} class I restricted dominant epitope and an I-A\textsuperscript{b} restricted class II dominant epitope. Processing and presentation of the class I and class II epitopes by the OVA mRNA-transfected DC was determined using T-hybridomas specific to each epitope. No presentation of the class II epitope was seen with a truncated form of OVA (tOVA) from which the first 40 aa containing the leader sequence were removed (Fig. 2A). This is consistent with the observations that class II presentation of endogenous antigens is confined primarily to antigens which can access the endocytic compartments\textsuperscript{14}. In contrast, the native secreted form of OVA is capable of processing the class II epitope for presentation to the class II restricted T-hybridoma. Incubation of the OVA mRNA transfected DC with an Ii AS, but not control, ODN enhanced the presentation of the class II OVA epitope. Presentation of the dominant OVA class I epitope was not significantly affected by the antisense or control ODN
(Figure 2B). This experiment shows that transient and partial inhibition of invariant chain expression in cultured DC enhances the presentation of class II, but not class I, epitopes from the endogenously expressed OVA antigen.

Figure 3

To determine whether the antisense ODN-mediated inhibition of invariant chain expression can lead to enhanced stimulation of OVA-specific CD4+ and CD8+ cytotoxic T cell (CTL) responses in vivo, we immunized mice with OVA mRNA-transfected DC treated with control or Ii AS ODN and the induction of OVA specific CD4+ T cell and CTL responses was measured in the
splenocytic population of the immunized mice. Only mice immunized with Ii AS ODN treated DC exhibited an OVA specific CD4\(^+\) T cell response, namely the CD4\(^+\) T cells proliferated against OVA, but not VSV, pulsed splenocytes (Fig. 3A). Influenza matrix (Flu M1) mRNA transfected DC, OVA mRNA-transfected DC alone or treated with a control ODN did not stimulate detectable levels of CD4\(^+\) T cell responses above background. The high basal level of CD4\(^+\) T cell proliferation seen in this experiment represents an anti-FCS response commonly seen when immunizing with DC cultured in the presence of FCS\(^{36}\). Attempts to reduce background by growing the DC or CD4\(^+\) T cells in syngeneic mouse serum or AIM-V media was unsuccessful. Nevertheless, the small difference in the proliferative capacity of the Ii AS ODN treated mice against OVA targets shown in Figure 3 was seen reproducibly in three experiments. Following in vitro stimulation of splenocytes from mice immunized with OVA mRNA transfected DC, an enhanced CTL was seen if the DC were treated with the Ii AS, but not with control, ODN (Fig. 3B). When the immunizing DC were treated with Ii AS ODN an OVA-specific CTL could be detected directly without ex vivo stimulation, thus underscoring the enhancing effect of invariant chain inhibition on CTL induction in vivo. When CTL analysis was performed 35 days post immunization the OVA-specific CTL activity was significantly diminished, yet in mice immunized with the Ii AS ODN treated DC the decrease in OVA CTL activity was less pronounced (Fig. 3C). 55 days post immunization OVA-specific CTL could not be detected in the in vitro stimulated splenocytes (data not shown). However, when mice were reimmunized with OVA mRNA transfected DC (not treated with Ii AS ODN) 85 days post initial immunization, the mice which received Ii AS ODN treated DC demonstrated a superior CTL response (Fig 3D). These observations show that immunization with Ii AS ODN treated DC extend the persistence of CTL precursors in the spleen of the immunized mice (Fig. 3C) and induce a larger pool of memory CTL which can be reactivated by immunization (Fig. 3D).
In the next series of experiments we tested whether antisense mediated inhibition of invariant chain synthesis in DC can enhance antitumor immunity. Mice (n=5) were immunized with OVA mRNA-transfected DC and challenged subcutaneously with OVA-expressing B16/F10.9 melanoma tumor cells. Figure 4A shows tumor sizes at day 21 post tumor challenge. Individual tumor measurements show a considerable intra-group variability. Nevertheless, significant differences were seen among the various treatment groups. Tumor growth was inhibited in mice immunized with OVA mRNA-transfected DC as compared to mice immunized with influenza M1 mRNA transfected DC or injected with PBS (p=0.002). Treatment of the OVA mRNA-
transfected DC with either of two Ii AS ODNs significantly enhanced the antitumor effect whereas neither of two control ODNs had any effect (p=0.002). As shown in Figure 4B, treatment of OVA mRNA transfected DC with the Ii AS ODNs significantly delayed the appearance of palpable tumors compared to OVA mRNA transfected DC treated with control ODNs (p=0.0001). Mice were monitored until day 42 days at which time the experiment was terminated.

To examine the mechanism underlying the invariant chain inhibition-mediated enhancement of tumor immunity, mice immunized with OVA mRNA-transfected DC were depleted of either CD4+ or CD8+ T cells prior to challenge with B16/F10.9-OVA tumor cells. Tumor growth in mice vaccinated with OVA mRNA-transfected DC expressing normal levels of invariant chain (DC treated with control ODN) was enhanced in CD4+ T cell depleted mice whereas depletion of CD8+ T cells had no effect (Fig. 5). CD4+ T cells are, therefore, the primary effectors of tumor inhibition in this model as has been seen in other studies9-11. Notably, depletion of CD8+ T cells abrogated the Ii AS ODN effect. This observation suggests that the antisense mediated inhibition of invariant chain expression enhances tumor inhibition by inducing a CD4+ T cell-dependent CD8+ T cell response. Additional studies using several tumor models will be required to determine the generality of this observation. It will be also important to determine the role of CD4+ and CD8+ T cells at the induction phase, by depleting the T cell subsets prior to immunization.
In the experiments shown in Figures 4 and 5 mice were immunized prior to challenge with tumor. To test whether invariant chain inhibition can also augment protective immunity in tumor bearing mice, mice were first implanted with B16/F10.9-OVA tumor cells and immunized with OVA mRNA-transfected DC starting three days following tumor implantation. Tumor growth was measured by monitoring the time to appearance of palpable tumors in the various treatment groups (Figure 6). Experiments were terminated after statistical significance was reached (days 41-43). A significant retardation in tumor growth was seen when mice were immunized with OVA mRNA-transfected DC, alone or treated with a control ODNs, compared to mice immunized with Flu M1 mRNA transfected DC (Fig. 6A, p= 0.0015). Inhibition of tumor growth was further enhanced when the OVA mRNA-transfected DC were treated with the Ii AS ODNs (p=0.006). To test whether Ii inhibition can also potentiate antitumor immunity directed against
natural tumor antigens, mice were immunized with TRP-2 mRNA or total B16/F10.9 tumor RNA. TRP-2 is a melanocyte specific ER-resident dominant tumor antigen in the B16/F10.9 melanoma tumor\textsuperscript{37}. As shown in Figure 6B, the antitumor effect seen in tumor bearing mice immunized with either TRP-2 mRNA (p=0.0535) or B16/F10.9 tumor RNA transfected DC (p=0.03) was enhanced when the DC were treated with Ii AS, but not control, ODNs. Treatment of influenza matrix (Flu M1) mRNA transfected DC with Ii AS ODNs had no significant antitumor effect (Fig. 6A, p=0.9 and data not shown), suggesting that the effect of invariant chain inhibition was OVA antigen-specific. This, and the observation that Ii AS ODN treatment of DC transfected with tOVA mRNA, which is not capable of presenting class II epitopes (Fig. 2), does not enhance tumor immunity (data not shown), argues against the contribution of FCS-derived antigens used in the DC generation protocol to the observed antitumor effects.
Figure 6

A.

![Graph A]

B.

![Graph B]
DISCUSSION

In this study we describe a simple and general method to enhance the stimulation of CD4+ T cell responses by DC presenting endogenous antigens. Using DC transfected with mRNA encoding the chicken ovalbumin (OVA), we have shown that a partial inhibition of invariant chain expression mediated by a short treatment with antisense oligonucleotides leads to enhanced presentation of class II OVA epitopes in vitro and in vivo (Figs. 2 and 3A).

CD4+ T cells are required for the persistence and proliferation of activated CD8+ T cells and the generation of long-lived memory CD8+ T cells7. The CD4+ T cell arm of the cellular immune response is also a critical component of an effective antitumor response5,6. Here we show that enhanced activation of OVA-specific CD4+ T cells in mice immunized with DC expressing reduced levels of invariant chain is accompanied by increased induction of CD8+ CTL (Fig. 3B), extended persistence of a measurable CTL response (Fig. 3C) and a larger pool of memory CTL that can be reactivated in vivo (Fig. 3D). In addition, inhibition of invariant chain synthesis in DC correlates with enhanced tumor immunity (Figures 4-6). This was seen reproducibly using DC transfected with either mRNA encoding the strong model antigen OVA, the endogenous TRP-2 tumor antigen, or unfractionated tumor-derived antigenic mixtures, in both prophylactic (Figs. 4 and 5) and therapeutic (Fig. 6) immunization protocols. Overall, these experiments underscore the important role of the CD4+ T cell arm in tumor immunity and suggest that induction of CD4+ T cell responses by immunization with mRNA-transfected DC is a limiting factor in stimulating antitumor immunity.

Invariant chain inhibition-mediated enhancement of class II presentation requires the translocation of endogenous antigens into the ER/endosomal compartment14 which can be achieved by appending a lysosomal targeting signal to the carboxy end of the antigen15. Whereas
the lysosomal targeting approach is limited to defined antigens that have to be modified individually with the lysosomal targeting signal, the invariant chain inhibition strategy described here will be also applicable when undefined cell (tumor) derived antigenic mixtures are used, as illustrated in the case of immunotherapy with B16/F10.9 melanoma tumor RNA transfected DC (Fig. 6). Conceivably, in this instance inhibition of invariant chain expression leads to enhanced stimulation of CD4⁺ T cell responses against a subset of tumor antigens, including perhaps TRP-2, which can access the endosomal compartment.

The strategy to enhance CD4⁺ T cell immunity described in this study is by no means limited to mRNA-transfected DC and should be equally useful when used in conjunction with other antigen loading techniques such as DC-tumor fusions, transduction of DC with viral vectors or incubation with apoptotic bodies or whole tumor cells.
REFERENCES


FIGURE LEGENDS

Figure 1: Inhibition of invariant chain expression (CD74) in DC incubated with antisense oligonucleotides.

A. Day 7 DC generated from the bone marrow of C57BL/6 mice were electroporated with OVA mRNA and 50 µM of Ii AS ODN (AE40) (filled histogram) or control ODN (SE46) (open histogram). Cells were replated, cultured for 2 days and stained with FITC-labeled anti-mouse CD74 (invariant chain) Ab. Dotted lines-DC stained with FITC-labeled isotype control Ab. To measure total invariant chain expression, cells were permeabilized before Ab staining. B. Two color staining of DC with PE-labeled anti-CD11c and FITC-labeled anti-CD74, CD40, CD80, CD86 or MHC class II (I-A<sup>b</sup>) Ab (PharMingen, San Diego, CA). Data is representative of 5 experiments.

Figure 2: Inhibition of invariant chain synthesis enhances MHC class II presentation of OVA by DC transfected with OVA mRNA.

DCs were transfected with OVA mRNA or a truncated OVA mRNA (tOVA) from which sequences corresponding to the first 40 aa of the OVA protein were deleted. As indicated, the OVA mRNA transfected DC were also treated with Ii AS (AE40) or control (SE40) ODN. Presentation of the dominant MHC class II (panel A) and class I (panel B) OVA epitopes was determined by measuring IL-2 secretion from OVA class II and class I T-hybridomas, respectively. Data is representative of 6 experiments using two Ii AS and control ODNs described in Materials and Methods section.
Figure 3: Inhibition of invariant chain synthesis enhances the generation of CD4⁺ T cell responses and cytotoxic T cell (CTL) responses in mice immunized with OVA mRNA transfected DC.

A. CD4⁺ T cell proliferation assay. Mice were immunized i.v. with 2.5x10⁵ DCs transfected with either OVA mRNA or influenza matrix (Flu M1) mRNA. Where indicated, the OVA mRNA transfected DC were also transfected with Ii AS (AE40) or control (SE40) ODNs. Splenocytes were harvested after 8 days and CD4⁺ T cells were isolated using StemSep Murine CD4⁺ Negative Isolation Column (StemCell Technologies Inc.). CD4⁺ T cells were co-cultured with I-Aᵇ-restricted OVA or VSV peptide pulsed DC for 3 days. ³H-thymidine incorporation was measured for 17 hr prior to harvest. Data are representative of 3 experiments.

B. Cytotoxicity assay. Mice were immunized with 2.5 x 10⁵ OVA mRNA-transfected DCs transfected with Ii AS (AE40) or control (SE40) ODNs, as indicated. Splenocytes isolated 8 days post immunization were either tested directly for OVA CTL (1⁰ response) or first incubated in vitro in the presence of OVA mRNA transfected DC and then tested for OVA CTL (2⁰ response)²⁹. RMA-S cells pulsed with the MHC class I restricted OVA or VSV peptides were used as targets. Data are representative of 5 experiments.

C. CTL responses measured 8 days or 35 days post immunization. D. 85 days after immunization with Ii AS (AE40) or control ODN (SE40) treated OVA mRNA-transfected DC, mice were reimmunized with OVA mRNA transfected DC and OVA CTL measured.

Figure 4. Enhancement of antitumor immunity in mice immunized with Ii AS ODN-treated DC.

Mice were immunized twice weekly with DC transfected with either OVA or Flu M1 mRNA, or mock immunized with PBS, treated with either of two Ii AS ODNs (AE54 or AE40) or two
control ODN (SE46 or SE54), as indicated, and challenged subcutaneously with B16/F10.9-OVA tumor cells 10 days after the second immunization (5 mice per group). A. Day 21 tumor measurements There was no statistical difference between the PBS group relative to the Flu M1 RNA mRNA group, among the three OVA mRNA groups not treated or treated with either of the two control ODNs, and between the two OVA mRNA groups treated with either of the two Ii AS ODNs. The P values for the PBS group relative to the OVA mRNA+control ODN groups, and for the OVA mRNA+control ODN groups relative to the OVA mRNA+Ii AS ODN groups was less than 0.002. B. Time to appearance of palpable tumors. Statistical significance was determined using the logrank test. P values were 0.0015 for the OVA mRNA+control ODN group relative to the Flu M1 RNA group, and 0.001 for the OVA mRNA+Ii AS ODN groups relative to the OVA mRNA+control ODN group. There was not statistical difference between the two OVA mRNA groups treated with either of two Ii AS ODNs. The median time to tumor onset was 9 days for the Flu M1 group, 17 days for the control OVA mRNA+ODN group and 30 days for the OVA mRNA+Ii AS ODN treated groups. Data are representative of 3 experiments.

**Figure 5:** The role of CD4⁺ and CD8⁺ T cell subsets in the antitumor response in mice immunized with DC expressing reduced invariant chain levels.

A. Role of CD4⁺ and CD8⁺ T cell subsets. Mice were immunized with OVA mRNA transfected DC and challenged with B16/F10.9-OVA tumor cells as described in Figure 4A. Mice were depleted of CD4⁺ or CD8⁺ expressing cells before tumor challenge, as indicated. Mice immunized with Flu M1 RNA or treated with PBS also received isotype Ab. Day 25 measurements are shown (5 mice per group).
Figure 6: Enhancement of tumor regression in mice immunized with Ii AS ODN-treated DC.

A. B16/F10.9-OVA tumor cells were implanted subcutaneously in C57BL/6 mice followed 3 days later by treatment with OVA or influenza matrix (M1) mRNA-transfected DC exposed to Ii AS (AE40) or control (SE40) ODNs as indicated (5 mice per group). Mice were monitored for the appearance of palpable tumors. Using the logrank test, the P values were 0.006 for the Ii AS ODN group relative to the control ODN group, and 0.0015 for the control ODN group relative to the Flu M1 group. There was not statistical difference between the OVA mRNA relative to the OVA mRNA+control ODN groups, or the Flu M1 mRNA group relative to the Flu M1 mRNA+Ii AS ODN group (p=0.9). The median time to tumor onset was 8 days for the Flu M1 with or without control ODN groups, 16 days for the OVA mRNA group, 16 days for the OVA mRNA+control ODN group and 35 days for the OVA mRNA+Ii AS ODN group. B. As in panel A, except that B16/F10.9 tumor cells were used and mice were immunized with either TRP-2 mRNA or B16/F10.9 tumor RNA-transfected DC. P values by the logrank test were 0.0535 for the TRP2 mRNA+Ii AS ODN group relative to the TRP2 mRNA+control ODN group, 0.03 for the B16 RNA+Ii AS ODN group relative to the B16 RNA+control ODN group, 0.0563 for the TRP2 mRNA group+control ODN group relative to the Flu M1+control ODN group and 0.0079 for the B16 RNA+control ODN relative to the Flu M1 group. The median time to tumor onset was 8 days for the Flu M1 group, 18 days for the B16 RNA+control ODN group, 15 days for the TRP2 mRNA+control ODN group, 21 days for the B16 RNA+Ii AS ODN group and 25 days for the TRP2 mRNA+Ii AS ODN group. Data are representative of 2 experiments.
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