Cross-linking a mAb to NKR-P2/NKG2D on dendritic cells induces their activation and maturation leading to enhanced anti-tumor immune response

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Abstract
NKR-P2/NKG2D is the chief tumor recognition receptor of NK cells and some T cells, which recognizes stress inducible ligands on tumors and mediates cell activation. We have recently reported the involvement of NKR-P2 in rat dendritic cell (DC) activation. We demonstrate the potential of agonistic anti-NKR-P2 mAb (1A6), which mimics the NKR-P2 ligand and induces activation and maturation of DCs. Interaction of DCs with 1A6 enhances nitric oxide-mediated apoptosis in tumor cells. NKR-P2 cross-linking with 1A6 also induced the secretion of inflammatory cytokines, IL-1β, tumor necrosis factor-α, IFN-γ and IL-12 by DCs. Blocking of 1A6-mediated activation and maturation with inhibitors of PI3K, p38K and ERK1/2K suggests involvement of MAP kinase in signal transduction. 1A6 cross-linking activates nuclear factor kappa B, which acts as key executor of DC activation. Administration of 1A6 antibody induces rapid regression and protective immune responses against transplantable tumors, suggesting mAb induced activation and maturation of DCs, leading to enhanced anti-tumor immune response.

Introduction
The activation of anti-tumor immune response has been well documented in tumor models and the role of effector immune cells has been extensively studied in tumor regression. However, in the presence of exogenous stimulation, the effective immune response could be enhanced to achieve a successful therapeutic effect. Dendritic cells (DCs) have emerged as a promising tool in cancer immunotherapy. In vivo, DCs are found at various anatomical locations as different subsets and maintain immunological homeostasis by sampling self-antigens (1). DCs exert an innate direct cytotoxic effect on tumor cells (2–6), through TRAIL, FasL, lymphotixin γδ, NKR-P1 receptors (7, 8) and also possess anti-tumor adjuvant function due to their specific T cell regulatory capacity (9), that is generated by the presentation of tumor antigen upon DC maturation (10). DC maturation is characterized by the modulation of distinct surface receptors and differential capacity of antigen processing and presentation (1). Activation- and maturation-associated fine reprogramming of DCs against neoplastic cells is exerted through various stimuli (11, 12). Therefore, stimuli dependent concerted anti-tumor responses have evolved to include DCs as potential immunotherapeutic agent.

Rat cytotoxic DCs also express NKG2D/NKR-P2-activating receptor (13). NKG2D is a homodimeric, type II c-type lectin-like receptor and expressed on the cell surface of NK cells, CD8αβ T cells, γδT, CD4+ T cells (14), mouse IKDCs (18, 15) and human myeloblastic KG1a cells (16). Recently, NKR-P2 transcript is also demonstrated in rat CD4+/CD8+ monocytes and macrophages (17). NKR-P2/NKG2D recognizes various stress inducible ligands on tumor cells and generates effective immune response (19). Previously, we have reported maturation of splenic dendritic cells (SDCs) after co-culture with fixed AK-5 tumor cells (20), involvement of NKR-P2 in the induction of DC-mediated anti-tumor immune response (13). Rat ortholog of mice and human NKG2D receptor is called NKR-P2; we have cloned rat-Rae-1-like transcript ligand from AK-5 tumor cells that interacts with NKR-P2 on rat NK cells (21).

Enhancement of immune response by cross-linking specific receptors on immune cells has emerged as a potential therapeutic tool in cancer treatment (22). Antibody-mediated activation and maturation of DC have been reported by cross-linking Fcγ, CD32, CD38, CD40, CD43, CD95, MHC II and B7-DC receptors (23–31). In the present work, we show...
that cross-linking of NKR-P2 with 1A6 on rat DCs provides a strong stimulatory signal, which in turn enhances apoptosis in tumor targets through nitric oxide (NO). 1A6-mediated DC activation is dependent on p38, PI3K, ERK1/2, protein kinase C (PKC), nuclear factor kappa B (NFkB) translocation and Ca\(^{2+}\) mobilization. Interestingly, 1A6 also transmits the effective maturation signal in DCs as characterized by enhanced MHC II, B7-2, CD1a expression, antigen-presentation function and reduced endocytic capacity of DCs. Besides, 1A6 also induces DCs to secrete TNF-α, a potent immunoregulatory cytokine. AK-5 tumor is a highly immunogenic rat histiocytoma and regresses spontaneously upon subcutaneous (s.c.) transplantation in syngeneic animals (32) whereas it kills 100% hosts by NK cell depletion upon intra-peritoneal (i.p.) transplantation (33). 1A6 administration causes activation and maturation of DC in vivo, resulting in faster regression of AK-5 tumor. The present study demonstrates the stimulatory potential of anti-NKR-P2 mAb, which could be utilized to enhance the therapeutic capability of DCs for successful treatment of cancer.

Methods

Animals and tumors

Inbred colony of Wistar rats of either sex of 5–7 weeks of age were used in this study. AK-5 tumor was maintained as ascites by injecting \(5 \times 10^6\) AK-5 cells i.p. and solid tumors were obtained by injecting \(3 \times 10^6\) AK-5 cells s.c. BC-8 (in vitro adapted single-cell clone of AK-5 tumor) was maintained in vitro in IMDM supplemented with 10% FCS. ZAH tumor (hepatoma) was maintained as ascites in wistar rats (i.p.). For in-vivo experiments, tumor cells were pooled from respective hosts. All animal experiments were done following the institutional guidelines and with the approval of the animal ethics committee.

Generation and isolation of DCs

Bone marrow cells were obtained from femur of rats. After RBC lysis (hypotonic shock with 0.84% NH\(_4\)Cl), the bone marrow cells were plated in culture medium (IMDM, 10% heat-inactivated FCS, 100 U ml\(^{-1}\) penicillin and 50 μg ml\(^{-1}\) streptomycin) for 1 h for the removal of resident macrophages. The non-adherent cells were collected and replated in complete medium supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) [obtained from GM-CSF-secreting CHO cells, GM-CSF content >10 ng ml\(^{-1}\) and mouse recombinant IL-4 (R&D system, 5 ng ml\(^{-1}\))] for 6 days to generate immature DCs. Rat bone marrow-derived dendritic cells (BMDCs) thus obtained were observed to be CD42, CD11c, MHC II and CD1a positive, <2–3% attached cells were positive for CD41 (SIRP). Similar procedure was followed to generate mouse BMDCs. Similarly, spleens were removed aseptically from experimental animals and teased in cold PBS. Total splenocytes were obtained by Ficoll–Hypaque density gradient, washed and plated for 30 min for macrophage (attached cells) elimination. The non-adherent cells were collected and SDCs were isolated using Dynal magnetic beads coated with CD41 mAb as per the manufacturer’s description (Dynal, Chantilly, VA, USA). For phenotypic characterization, both SDCs and peritoneal dendritic cell (PDCs) were immediately fixed with 2% PFA after magnetic bead (OX-62) separation.

Total peritoneal cells were obtained by injecting cold PBS in the peritoneal cavity and re-suspended in IMDM medium supplemented with 10% heat-inactivated FCS, and cultured in 100 mm petri dishes for 1 h at 37°C. The non-adherent cells were removed by gentle washing with warm IMDM (37°C) and the adherent cells i.e. DC and M\(_\phi\) were cultured for additional 10 h at 37°C, whereas most M\(_\phi\)s remained attached after this period, and DCs became non-adherent. The non-adherent fraction was further enriched for DC with mAb OX-62-coated magnetic beads (Dynal). Using the above-mentioned protocol, we obtained ~95% pure SDCs and PDCs as assessed by morphology and phenotypic markers (OX-62, CD11c and MHC II). Peritoneal adherent macrophages were isolated by aspirating peritoneal cavity of experimental animals with chilled PBS followed by removal of floating cells after 1h, similarly adherent splenic macrophages were obtained from mononuclear lymphocytes excluding SDCs (after magnetic bead separation for DC with OX-62) by removal of floating cells after 30 min.

Isolation and priming of CD8\(^+\) T cells

BALB/c mice (8 weeks) were with injected ovalbumin antigen (2 mg per animal) for priming of CD8\(^+\) T cells. After 7 days, total splenocytes were isolated by Ficoll density centrifugation. CD8\(^+\) T cells were isolated by a two-step positive-selection procedure (anti-CD3 followed by anti-CD8-coated dynal beads). Afterward, CD8\(^+\) T cells were incubated with rIL-2 (human rIL-2, 5 ng ml\(^{-1}\)) for 48 h and the cells were harvested for staining. Similarly, rat CD8\(^+\) T cells were isolated by a two-step isolation procedure as described above.

Antibodies and reagents

Hybridoma OX-62 (2α-integrin) was kindly provided by M. J. Puklavac, clone GL1 (B7-2) by Vijay Kuchroo, anti-mouse IL-12 (C17.15, p40 subunit) by G. Trinchieri, anti-mouse IFN-γ (XMG1.2) by R. L. Coffmann and anti-ICAM-1 (clone IA29) was provided by M. Miyaska. Anti-CD8 (OX-8), anti-tumor necrosis factor-α (TNF-α) and anti-IL-1β were from BD Biosciences. Anti-mouse, anti-rabbit, anti-goat and anti-rat Ig Alexa 488 were from Molecular Probes. Human rIL-2 and ovalbumin were from R&D system and Sigma, respectively.

A hybridoma secreting agonistic mAb1A6 (IgM) against NKR-P2 was raised in BALB/c mouse in our laboratory (13). Clone 3C6 (IgM), a non-agonistic anti-NKR-P2 mAb, was used as internal negative isotype control throughout the study. mAb1A6 was tested for the presence of endotoxin using limulus amebocyte lysate assay (Biowhittaker) within the sensitivity level of 0.125 EU. Endotoxin levels were always below the permissible limits (10 pg mg\(^{-1}\)).

Anti-mouse iNOS was from BD Transduction Laboratory. Rabbit anti-mouse NFkB (p65 subunit), rabbit anti-mouse FasL, goat anti-mouse NKG2D and IκB-α were from Santa Cruz, anti-human CD16, anti-CD3 and anti-E1d1 were from SeroTec. Antibodies for total p38, pp38, AKT, pAKT, ERK1/2 and pERK1/2 were from BD Biosciences. MHC II, CD11c and CD1a (FITC tagged) were from BD Biosciences. LPS
DC maturation with anti-NKR-P2 antibody

Tumor growth inhibition, apoptosis assay, flow cytometry and immunostaining

Anti-tumor cytostatic activity of DCs was determined by the inhibition of DNA synthesis in target tumor cell. Briefly, BMDCs, SDCs or PDCs obtained after stimulation (in vivo or in vitro) were co-cultured with non-adherent BC-8 tumor cells at experimental ratio for 24 h. To estimate the DNA synthesis, cells were pulsed with [3H]thymidine ([3H]TdR) (1 μCi per well) during the last 10 h of incubation. [3H]TdR incorporation was determined by measuring the radioactivity incorporated in BC-8 cells and the results are expressed as counts per minute for triplicate wells ± standard error of the mean.

Adherent BMDCs, SDCs or PDCs were activated with mAb1A6 for 4 h in the presence or absence of W1400 along with relevant control and co-cultured with BC-8 cells at 5:1 ratio. Induction of apoptosis in tumor cells was assessed by FACS after propidium iodide (PI) staining of fixed BC-8 cells and Annexin V staining after 16 h co-culture as judged by MTT assay.

DCs were fixed in 2% PFA for surface staining and probed with MHC II, B7-2 and CD1a (FITC tagged) antibodies, followed by appropriate secondary antibodies. Stained cells were analyzed on FACS (Becton Dickinson).

Isolated SDCs and BMDCs were plated on cover slips, the cells were fixed with 2% PFA and probed with goat anti-mouse NKG2D polyclonal antibody (20 μg ml⁻¹) and mouse CD11c mAb for 12 h at 4°C, after washing the respective primary antibodies, the cells were probed with appropriate secondary antibody. The scanning was performed on LSM Carl Zeiss confocal microscope.

Estimation of NO

DCs (BMDCs, SDCs or PDCs) were incubated at 2 × 10⁶ cells per well (triplicate) in 96-well plates along with different activators in IMDM (Invitrogen) supplemented with 10% FCS for indicated time. Cell-free culture supernatants were recovered after incubation and NO content was measured with Griess reagent. The absorbance at 540 nm was measured using ELISA reader (Molecular Devices, Spectra Max 190). Nitrite content was quantified from standard curve generated using sodium nitrite.

Reverse transcription–PCR and western blotting

Total cellular RNA was isolated using Trizol reagent. cDNA was prepared using oligo(dT) 12–18 primer and AMV Reverse Transcriptase (Promega). Semi-quantitative reverse transcription (RT)–PCR was carried out using specific primers for iNOS, IL-12, TNF-α, IFN-γ and IL-1β along with GAPDH primers.

BMDCs were stimulated with 1A6 or LPS for indicated time and lysed in Laemmlli buffer. Lysates were resolved on 10% SDS–PAGE, the proteins were transferred to nitrocellulose followed by immunoblotting with anti-mouse iNOS mAb and equal loading was confirmed by amplification of β-actin signal. Similarly, immunoblotting was performed with pp38, pAKT and pERK1/2 and equal loading was confirmed by total p38, AKT and ERK1/2-specific mAbs. Total cellular extract was used to detect iKB-α with polyclonal antibody and equal loading was checked with β-actin. Nuclear extract from the stimulated cells was probed with polyclonal NFkB antibody and the equal loading was confirmed by Ponceau S stain.

Enzyme-linked immunofiltration assay (cytokine assay)

IL-12, TNF-α, IFN-γ and IL-1β levels in culture supernatants were measured by ELIFA (Pierce Co., Rockford, IL, USA). Briefly, culture supernatants were filtered through nitrocellulose membrane, which traps the cytokines/proteins specifically, followed by blocking with BSA (3%) and capturing with appropriate mAbs. HRPO-clubbed signal was infiltrated with color sensitive substrate in 96 well and read at 490 nm along with negative and positive controls.

MLR assay

For the mixed lymphocyte reaction (MLR) assay, T lymphocytes were enriched by nylon wool followed by anti-CD3 mAb-coated Dynal beads. The purity of T cells was >95% as assessed by FACS using anti-CD3 mAb. After 24 h stimulation with 1A6, attached experimental BMDCs (blocked with rNKR-P2 protein) were washed and cultured at 1:20 ratio with autologous T lymphocytes for 3 days, and further treated with 1A6. Proliferation was measured after 3 days. At 18 h before termination, 1.0 μCi [3H]Tdr was added per well. Cells were harvested and incorporated radioactivity was measured in a β-counter.

Quantitation of endocytosis

BMDCs and SDCs were stimulated with mAb1A6 for 24 h, washed, re-suspended in complete media and incubated with Zymosan–FITC (1 mg ml⁻¹) for 1 h at 37°C. Uptake was stopped by washing the cells with chilled PBS containing 0.01% NaN₃. Cells were washed four times and analyzed with FACS. Surface-binding values obtained by incubating cells at 4°C were subtracted from the values measured at 37°C.

Cell aggregation assay

Non-adherent BMDCs and SDCs were incubated with isotype control mAb and 1A6 for 2 h and photographed at different fields to score extent of homotypic clustering.

AK-5, ZAH tumor growth studies

Male Wistar rats (4–6 weeks, five animals per group) were challenged with tumor cells s.c. (3 × 10⁶ cells per animal) and were given nine antibody injections (1A6 1 mg ml⁻¹, i.p. or s.c.) on alternate days. Isotype-matched control antibody was used in control group. Tumor growth was monitored on different days. Wistar rats were given twelve 1A6 injections for AK-5 ascites and six 1A6 injections for ZAH ascites. Animals were routinely monitored for host death and tumor growth.
Results

Activation of DCs with mAb1A6 in vitro

NKR-P2/NKG2D-expressing rat NK cells, RNK-16 cells, CD8+ T cells and BMDCs were stained with mAb1A6, which showed significant staining of NKR-P2 on rat BMDCs (Fig. 1A). Similarly, mouse NK cells, activated CD8+ T cells and BMDCs were stained with mAb1A6 and polyclonal anti-NKG2D antibody (Fig 1B). NKR-P2/NKG2D expression was also confirmed by staining of rat BMDCs and SDCs on CD11c+ DCs (Supplementary Figure, available at International Immunology Online). We also examined the phenotypic profile of freshly isolated SDCs and PDCs to assess the purity of the cell proportions (Fig. 1C). We further determined the proportion of DCs, which expressed NKR-P2 receptor. OX-62-stained BMDCs, PDCs and SDCs were positive for NKR-P2 after staining with mAb1A6 (Fig. 1D). In order to check the activation of DCs from different sites with agonistic anti-NKR-P2 mAb (1A6), pure populations of BMDC, PDC and SDC were incubated with isotype control antibody, mAb1A6, fixed BC-8 tumor cells and LPS for 24 h. NO produced in response to NKR-P2 cross-linking from BMDCs, PDCs and SDCs was estimated. About 6- to 7-fold increase in NO levels was observed with BMDCs and PDCs, whereas ~2-fold increase was detected from SDCs in vitro. Co-culture of fixed BC-8 tumor cells with DCs (25:1) and LPS (1 µg ml−1) stimulation was used as positive controls in the assays. In parallel assays, peritoneal macrophages do not show activation with similar stimuli except LPS (Fig. 1E). We also determined if in vivo administration (i.p.) of mAb1A6 induced the activation of PDCs and SDCs. PDCs and SDCs were harvested after 36 h of 1A6 injection and treated again with mAb1A6 as second dose of activator or left untreated for 24 h. About 6-fold increase in NO production was obtained from 1A6-injected PDCs, which increased further upon second dose of 1A6 stimulation in vitro. However, SDCs isolated from 1A6-injected animals produced ~2.5-fold more NO in comparison to control, whereas second dose of 1A6 (in vitro) to SDCs further enhanced the NO secretion significantly. LPS was also used as second activator (Fig. 1F and G). Isotype antibody did not cause any activation in vivo (data not shown). We have also estimated tumoristatic potential of 1A6-activated PDCs and SDCs. PDCs and SDCs from 1A6-injected rats were able to suppress growth of BC-8 tumor cells significantly in vitro (Fig. 1H and I). Growth of BC-8 cells alone was slightly inhibited in the absence of 1A6-treated DCs.

To illustrate mAb1A6-mediated DC activation and tumor cell killing, we have studied activation of immature BMDCs with 1A6. Figure 3(A) demonstrates up-regulation of iNOS transcript and iNOS protein upon 1A6 cross-linking on day 6 immature BMDCs. Induction of iNOS transcript and protein was not observed with isotype control antibody at similar concentrations. However, comparable iNOS induction was detected after co-culture with fixed BC-8 cells and LPS, which were used as positive controls.

As a result of iNOS induction, 6- to 7-fold increase in NO content was observed in 1A6-treated BMDCs. NO secretion was significantly abrogated upon pre-treatment of BMDCs with irreversible iNOS inhibitor W1400. Similarly, NO levels in BC-8 and LPS-treated BMDCs were also inhibited with W1400 (Fig. 3B).

Since NO is a well-known mediator of apoptosis in tumor cells, we assayed apoptosis in tumor cells co-cultured with 1A6-activated BMDCs by flow cytometry after PI staining and also by Annexin V staining. Interestingly, enhanced apoptosis in tumor cells was observed with mAb1A6-treated BMDCs. Apoptosis was significantly abrogated upon pre-treatment of BMDCs with W1400 before 1A6 stimulation. The presented data clearly elucidate NO as the potent mediator of DC-induced apoptosis in tumor cells (Fig. 3C). Tumor cell co-culture does not affect the viability of BMDCs as monitored by MTT assay (data not shown).

Furthermore, we also determined the cytostatic activity of 1A6-activated BMDCs. Figure 2(D) demonstrates cytostatic activity of BMDCs upon 1A6 cross-linking. Tumor cell proliferation was significantly inhibited upon NKR-P2 cross-linking, which was abrogated upon W1400 treatment. However, isotype control-treated and -untreated BMDCs inhibited slightly the growth of tumor cells as indicated in Fig. 3(D).

1A6-dependent cytotoxicity is mediated by NO

To determine whether mAb1A6 binds to NKR-P2/NKG2D and truly mimics its ligand, we performed the 1A6-binding assays upon NKG2D preblocking. Significant decrease in binding of 1A6 was observed with goat anti-NKG2D polyclonal antibody on SDCs (Fig. 2A). Similarly, a significant decrease in activating potential of 1A6 was also seen upon blocking the NKR-P2/NKG2D with bivalent anti-NKG2D polyclonal antibody as found by decreased NO production (Fig. 2B). Our earlier observation (13) shows that 1A6 acts as a blocking antibody for NK cells under in vitro conditions. We further cross-checked the NK cell activation with 1A6 in vivo, but could not find the up-regulation of FasL and IFN-γ on splenic NK cells (Fig. 2C). Similarly, iNOS and TNF-α levels remained unchanged in splenic macrophages upon 1A6 injection (Fig. 2D).

To rule out the possibility of endotoxin contamination in mAb1A6 preparation, we performed LAL assays and the endotoxin contamination was always below the permissible limit (<0.125 EU). Furthermore, boiling of 1A6 (100°C, 20 min) completely abrogates its activity whereas retains its potential upon polymyxin B (50 µg ml−1) treatment. In parallel assays, LPS activity was significantly abrogated with polymyxin B and remained unaltered upon boiling (100°C, 10 min) (Fig. 2E).

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the effect of PD98059, Ly294002 and SB203580 on iNOS levels. Significant inhibition of iNOS expression was observed upon pre-treatment of BMDCs with ERK1/2 inhibitor PD98059, AKT kinase inhibitor Ly294002 and only a slight down-regulation of iNOS was observed with p38 kinase inhibitor SB203580 (Fig. 4A).

Since NO is the final product of iNOS induction, we have measured NO content in PD98059-, Ly294002- and SB203580-treated BMDCs along with experimental controls and found a significant reduction in NO production with these inhibitors, which confirms the involvement of MAP kinase in 1A6-mediated BMDC activation. Significant inhibition of NO production was...
also observed with Ca\textsuperscript{2+} chelator EGTA, PKC inhibitor, H7 and with protein tyrosine kinase inhibitor, genistein (Fig. 4B).

We have also examined phosphorylation kinetics of p38, AKT and ERK1/2 upon 1A6 cross-linking and compared it with LPS-mediated signal transduction at similar time points by western blotting (Fig. 4C). Upon 1A6 stimulation, maximum pp38 was found at 45–60 min, whereas LPS caused significant phosphorylation at 10 min. Both 1A6- and LPS-mediated p38 phosphorylation was inhibited by imidazole SB203580. However, complete inhibition was observed in the case of 1A6 at 60 min. Chomone Ly294002 is a specific inhibitor of PI3 kinase and blocks activation of AKT kinase. pAKT form was significantly up-regulated at 45 min upon 1A6 cross-linking as well as by LPS treatment. Ly294002 completely abrogated the 1A6-mediated AKT phosphorylation at 60 min and a significant blocking was also observed in LPS-mediated activation.

The ERK pathway is known to be important in many cell types as regulator of cell survival. ERK1/2 phosphorylation was detected within 10 min after 1A6 or LPS stimulation. LPS induced stronger activation of ERK2 at 60 min and 1A6 induced ERK1 activation strongly till 45 min. Strong activation of both ERK1 and ERK2 was observed at 45 and 60 min with LPS, whereas significant ERK2 activation was observed only at 60 min with 1A6. However, flavanoid PD98059 inhibited significantly both ERK1/2 phosphorylation at 60 min (Fig. 4C).

NF\textsubscript{κ}B plays a significant role in DC activation; therefore we have investigated the role of NF\textsubscript{κ}B in mAb1A6-induced BMDC activation. The transcription factor NF\textsubscript{κ}B is bound to I\textsubscript{κ}B-\alpha in the cytoplasm and retained there in an inactive form. LPS stimulation results in degradation of I\textsubscript{κ}B-\alpha and as a result free NF\textsubscript{κ}B translocates to the nucleus and activates transcription of various genes. I\textsubscript{κ}B-\alpha degradation was
Fig. 2. (A) Inhibition of 1A6 binding upon NKG2D preblocking: SDCs were pre-blocked with anti-mouse NKG2D for 2 h, followed by staining with 1A6 (filled histogram, anti-mouse secondary antibody control; bold line, 1A6; thin line, 1A6 pre-blocked with anti-NKG2D and broken line, 1A6 pre-blocked with mouse IgG). Numbers indicate the respective mean fluorescence intensity for each staining. (B) Inhibition of 1A6 activity (decreased NO production) upon preblocking of NKG2D: BMDCs were pre-blocked with anti-mouse NKG2D followed by stimulation with 1A6 as indicated in the figure. (C and D) In vivo analysis of splenic NK cells and splenic macrophages upon mAb1A6 injection: staining of Fasl and intracellular IFN-γ for splenic NK and iNOS and TNF-α for splenic macrophages upon 1A6 injections (filled histograms represent secondary antibody controls and open histogram represent specific staining with respective antibody, number in figure represent the mean fluorescence intensity of fluorescence) (1 mg ml⁻¹ per rat day⁻¹—five injections each in three rats per group along with appropriate controls). (E) Anti-NKR-P2 mAb1A6 is free of endotoxin contamination: upon boiling agonistic nature of 1A6 was completely abrogated due to its proteinaceous nature, whereas polymyxin B failed to neutralize its agonistic nature. The standard assay (parallel assay with LPS) was also performed to ensure working status of the assay. Experiments performed describe the data obtained in three independent experiments.
Fig. 3. NKR-P2 cross-linking enhances cytostatic and cytotoxic activity of BMDCs through NO. (A) Up-regulation of iNOS transcript and protein upon NKR-P2 cross-linking. BMDCs were incubated with control mAb or mAb1A6 for 24 h. RNA and protein were isolated from similar experimental setup and subjected to RT–PCR (using iNOS-specific primers), and immunoblotting (rabbit anti-mouse iNOS antibody). GAPDH and β-actin were used to ascertain equal loading in RT–PCR and western. Fixed BC-8 cells (25:1) in co-culture and LPS (1 μg ml⁻¹) were used as positive controls. (B) NO secretion upon NKR-P2 cross-linking was inhibited with W1400. BMDCs were pre-treated with W1400 (5 μM, 45 min) and re-stimulated with mAb1A6. Fixed BC-8 cells (25:1) and LPS in the absence and presence of W1400 were also used. Cell-free culture supernatants were collected from triplicate experimental setup after 24 h and nitrite concentration was measured (mean ± SD) by Griess reagent. (C) NKR-P2 cross-linked BMDCs possess enhanced tumor cell apoptotic activity through NO. BMDCs were pre-treated with W1400 and stimulated with mAb1A6 for 6 h along with experimental controls as indicated in the figure. Subsequently, BMDCs were co-cultured with non-adherent BC-8 tumor targets at 25:1 ratio for 24 and 18 h to estimate hypoploidy and phosphatidyl serine externalization with PI and Annexin V staining separately. Upper panel shows DNA degradation and the percentage apoptotic tumor cells. Lower panel shows percentage Annexin V-positive BC-8 tumor cells in M1-marked open histograms. Filled histograms in the lower panel demonstrate autofluorescence (tumor cell co-culture does not affect the viability of BMDCs as monitored by MTT assay after co-culture experiment, data not shown). (D) NKR-P2 cross-linked BMDCs mediate cytostatic action through NO. BMDCs were pre-treated with W1400 and stimulated with mAb1A6 for 6 h along with experimental controls as indicated in the figure. Subsequently, BMDCs were co-incubated with live BC-8 tumor cells (doubling time 16 h) for 36 h. [³H]Tdr was added 10 h before tumor cell harvesting.
Fig. 4. A) Effect of PD98059, LY294002 and SB203580 on mAb1A6-induced iNOS expression. BMDCs were pre-treated with or without PD98059 (20 μM), SB203580 (10 μM) or LY294002 (50 μM) for 1 h before stimulation with mAb1A6 for 12 h. Cell lysates were analyzed by immunoblotting using rabbit anti-mouse iNOS antibody. (B) Effect of PD98059, Ly294002, SB203580, EGTA, H7 and genistein on 1A6-induced NO production. BMDCs in triplicate were incubated with PD98059 (20 μM), LY294002 (50 μM), SB203580 (10 μM), EGTA (2 mM), H7 (10 μM), genistein (10 μM) or medium alone for 1 h before stimulation with mAb1A6 (100 μg ml⁻¹). After a further 24 h incubation, culture supernatants were collected and nitrite content was measured. (C) 1A6 induces phosphorylation of p38 MAPK, AKT and ERK. BMDCs were treated with 1A6 (100 μg ml⁻¹) or LPS (1 μg ml⁻¹) separately and subjected to immunoblotting with specific mAbs. As indicated in the figure, BMDCs were pre-treated with SB203580 (upper panel), Ly294002 (middle panel) and PD98059 (lower panel) for 45 min before exposure to 1A6 and LPS. The panels show detection of phosphorylated form of p38, AKT and ERK1/2 along with unphosphorylated forms as loading controls at different time intervals along with inhibitors. (D) Role of NFκB in mAb1A6-induced BMDC activation. 1A6 induces the degradation of IκB-α in immature BMDCs. BMDCs were treated with 1A6 (100 μg ml⁻¹) and LPS (1 μg ml⁻¹) for different time points as indicated, followed by SDS–PAGE. IκB-α signal was detected with specific mAb, along with β-actin detection, as equal loading controls. In a similar experimental setup, nuclear extracts of BMDCs were subjected to immunoblotting with anti-NFκB p65 (lower panel). Ponceau S blot of nuclear extract is shown for equal loading of proteins.
observed within 30 min upon LPS treatment. However, significant degradation of iKB-α was only observed at 60 min upon 1A6 cross-linking indicating similar but slow activation of NFκB with 1A6 in comparison to LPS (Fig. 4D). To confirm NFκB translocation, nuclear extracts of stimulated BMDCs were immunoblotted with NFκB p65. Upon LPS stimulation, NFκB was detected at 30 min, whereas in 1A6-stimulated cells, the band was detected at 60 min (Fig. 4D). Hence, iKB-α degradation correlates very well with NFκB translocation and confirms the activational translocation.

1A6 induces maturation of DC and proliferation of autologous T cells

BMDCs and SDCs without any stimulus or with isotype control mAb showed the typical phenotypes of immature BMDCs and SDCs. However, upon NKR-P2 cross-linking with mAb1A6, both BMDCs and SDCs undergo maturation in 24 h. MHC II, B7-2 and CD1a-specific monoclonals detected enhanced surface expression of these maturation markers on 1A6-treated DCs. Average mean percentage of MHC II cells were 20 and 35% for BMDCs and SDCs, while 1A6 cross-linking increased this to 92 and 97%, respectively, in 24 h. Similarly, B7.2 levels were increased from 22 to 96% for BMDCs and 44 to 97% for SDCs. Upon 1A6 cross-linking, CD1a expression was also enhanced from 5 to 22% for BMDCs and 11 to 25% for SDCs. Co-culture of adherent BMDCs and SDCs with fixed BC-8 tumor cells at 1:25 ratio also induced up-regulation of MHC II, B7-2 and CD1a and was used as positive control to assess maturation (Fig. 5A). Similarly, LPS (1 μg ml⁻¹) stimulation also induced maturation of BMDCs and SDCs comparable to 1A6-induced maturation (data not shown). However, heat-denatured 1A6 failed to induce up-regulation of DC maturation markers (data not shown).

After evaluating the phenotypic maturation of BMDCs and SDCs, we tested the ability of BMDCs and SDCs to stimulate T cell proliferation in an autologous MLR assay. BMDCs and SDCs with isotype mAb exhibited a higher T cell proliferation activity. Upon 1A6 cross-linking, ~3-fold proliferation was detected with BMDCs, whereas ~4-fold proliferation was recorded with SDCs. Stimulatory capacity of 1A6 was significantly inhibited upon 1A6 neutralization with recombinant rNKR-P2 protein for both BMDCs and SDCs. rNKR-P2 protein did not induce stimulation of BMDCs (Fig. 5B). The T cell stimulatory capacity of SDCs was found to be higher than BMDCs. To exclude the possibility of a direct action of 1A6 on T cells, autologous T cells treated with 1A6 were also used as responder in MLR assay. However, 1A6 alone does not induce T cell proliferation directly (data not shown).

Role of p38 MAPK, AKT and ERK pathways in 1A6-mediated maturation of DCs

To investigate the role of MAPK-signaling pathway in 1A6-induced BMDC and SDC maturation, we pre-treated both BMDCs and SDCs with PD98059, LY294002 and SB203580 and evaluated expression profile of MHC II and B7-2 upon 1A6 treatment using double staining. A 25% reduction in MHC II–B7-2 expression was found with ERK1/2 inhibitor on BMDCs, whereas on SDCs it was inhibited by 30%. Ly294002 inhibited double staining to 37% on BMDCs and 20% on SDCs. SB203580 inhibited expression to 35% on BMDCs and 45% on SDCs (Fig. 6A). Taken together, these results suggest involvement of MAP kinase signaling in 1A6-mediated maturation.

1A6 cross-linking reduces endocytic capacity and increases clustering of DCs

Immature DCs possess high endocytic capacity whereas upon maturation, the endocytic function is reduced and the antigen-presentation function is modulated. We have evaluated the effect of 1A6 treatment on endocytosis by monitoring uptake of Zymosan–FITC in BMDCs and SDCs. At 4°C, the endocytic capacity is inhibited, however, at 37°C both untreated and isotype control-treated BMDCs showed significant uptake, whereas cross-linking of NKR-P2 with 1A6 inhibited the Zymosan–FITC uptake. A similar pattern of endocytosis was also observed with SDC. Similarly, in both BMDCs and SDCs, inhibition of endocytosis was observed upon coculture with fixed BC-8 tumor cells. LPS-matured BMDCs and SDCs were used as positive control in the assay (Fig. 6B). SDCs showed less uptake of Zymosan–FITC than BMDCs, probably because of their intrinsic maturation status.

Cellular aggregation in response to surface antigen signaling is a synchronized process and essential for cell–cell interaction and adhesion. Upon treatment of 1A6, both BMDCs and SDCs undergo homotypic clustering. BMDC cultures led to formation of large homotypic clusters, whereas SDCs showed comparatively smaller homotypic clusters in 6 h. This phenomenon was not observed with isotype-matched mAb (Fig. 6C). Clustering of BMDCs and SDCs was not observed upon LPS treatment (data not shown).

1A6 triggers synthesis of inflammatory cytokine by DCs

DC maturation stimuli are known to induce the secretion of inflammatory cytokines, such as IL-12, TNF-α, IFN-γ and IL-1β. Low concentration of cytokines was detected in the culture supernatants of BMDCs and SDCs. Upon 1A6 stimulation in vitro, significantly higher amounts of IL-12, IFN-γ and IL-1β were produced by BMDCs, whereas SDCs produced IL-12, IFN-γ, IL-1β and TNF-α also. Isotype-matched control mAb failed to stimulate BMDCs and SDCs under similar conditions. Upon co-culture with fixed BC-8 cells, significantly higher levels of these cytokines were produced by both BMDCs and SDCs (Table 1). Cytokine elevation was also confirmed by semi-quantitative RT-PCR for both BMDCs and SDCs (Fig. 7A).

Immunotherapeutic potential of 1A6 in anti-tumor responses

To evaluate the therapeutic potential of agonistic 1A6, we examined its anti-tumor activity in AK-5 tumor regression model. Rats were implanted with 3 × 10⁶ tumor cells at s.c. site and tumor growth was monitored till its complete regression. Interestingly, co-administration of 1A6 either by s.c. or i.p. along with 3 × 10⁶ tumor cells (s.c.) resulted in slow growth and faster regression of the tumor. 1A6-mediated rapid
Fig. 5. A) Induction of DC maturation markers with anti-NKR-P2 mAb. BMDCs and SDCs were incubated with medium alone, isotype control mAb, 1A6 and with fixed BC-8 cells at 25:1 ratio (BC-8:DCs) for 24 h. Cells were analyzed by FACS using anti-mouse MHC II, anti-rat B7-2, anti-mouse FITC-tagged CD1a and appropriate secondary antibody. Marker M1 (open histogram) represents the percentage positive cells along with appropriate secondary antibody control (filled histogram). The values shown are representative of three similar experiments. (B) Monoclonal 1A6 enhances the capacity of DCs to activate T cells. BMDCs and SDCs were incubated with 50 μg ml⁻¹ 1A6, 50 μg ml⁻¹ 1A6+ 50 μg ml⁻¹ rNKR-P2 and 50 μg ml⁻¹ rNKR-P2 protein for 24 h. Cells were harvested and plated in 96 well at 1:20 (DC:T) ratio. [³²P]Tdr incorporation was measured after 36 h. Isotype control mAb did not show significant autologous T cell proliferation. Results are expressed as mean counts per minute × 10³ ± SD, and are representative of three similar experiments.
regression of tumor was also found to be associated with the appearance of early regression spots in solid tumors (Fig. 7B). AK-5 is highly virulent upon i.p. transplantation and leads to 100% mortality of rats (34). 1A6 co-administration (i.p./s.c.) cured 60% of animals and showed delayed death in 40% rats, whereas all control animals died by early ascites development (Fig. 8A).

ZAH being less virulent, higher number ($2 \times 10^7$) of cells were transplanted to develop ascites in rats. A prolonged survival of host was seen upon 1A6 co-administration whereas all control animals died by day 10 (Fig. 8B).

These in vivo results demonstrate that 1A6 confers promising therapeutic potential against tumors of multiple origins ranging from slow growing solid tumor to ascitic tumor.

To investigate functional status of DCs in vivo, 1A6 was injected in normal rats. 1A6-injected OX-62-positive SDCs were found to possess enhanced expression of iNOS and B7-2 (Fig. 7C), which demonstrates higher activation status.
Table 1. Cytokine release upon NKR-P2 cross-linking with mAb1A6

<table>
<thead>
<tr>
<th>Cytokine (pg ml⁻¹ 10⁻⁶ cells)</th>
<th>IL-12</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMDC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.5 ± 1</td>
<td>17 ± 2</td>
<td>7 ± 3</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>Isotype</td>
<td>4.2 ± 2</td>
<td>20 ± 1</td>
<td>9 ± 2</td>
<td>18 ± 4</td>
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<tr>
<td>mAb1A6</td>
<td>77 ± 3</td>
<td>23 ± 4</td>
<td>80 ± 3</td>
<td>121 ± 3</td>
</tr>
<tr>
<td>BC-8</td>
<td>102 ± 8</td>
<td>82 ± 5</td>
<td>27 ± 7</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>SDC</td>
<td>7.3 ± 5</td>
<td>30 ± 2</td>
<td>18 ± 4</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Isotype</td>
<td>10.3 ± 2</td>
<td>32 ± 1</td>
<td>21 ± 7</td>
<td>16 ± 7</td>
</tr>
<tr>
<td>mAb1A6</td>
<td>54 ± 3</td>
<td>92 ± 18</td>
<td>121 ± 5</td>
<td>223 ± 16</td>
</tr>
<tr>
<td>BC-8</td>
<td>87 ± 1</td>
<td>120 ± 107</td>
<td>316 ± 8</td>
<td>240 ± 7</td>
</tr>
</tbody>
</table>

BMDCs or SDCs were incubated with 100 μg ml⁻¹ isotype control antibody, 1A6 for 24 h. Control DCs were left untreated or co-cultured with fixed BC-8 cells (25:1) ratio for 24 h and cytokine levels were measured in cell-free supernatant by enzyme-linked immunofiltration assay. Data are expressed as pg ml⁻¹ 10⁻⁶ cells ± standard deviation. *P < 0.05, student's t-test. Similar results were obtained in three independent experiments.

of DCs in vivo and therefore could be responsible for faster regression of AK-5 tumor.

Discussion

The interaction between NKG2D/NKR-P2 and its ligands is involved in anti-tumor immune responses, and the significance of this interaction is well studied for NK and CD8⁺ T cells (34). To generate specific anti-tumor immune responses, DCs directly kill the tumor cells and present the tumor-associated processed antigen upon maturation (10). We have recently shown the direct killing of tumor cells (13) and the maturation of DCs upon NKR-P2 interaction with its ligand on AK-5 tumor cells (Srivastava, R. M and Khar, A, unpublished observations). A prerequisite for successful treatment in cancer therapy is the establishment of innate and adaptive anti-tumor immune responses; in this regard immunotherapy is an attractive approach. Because of DCs' unique properties, much attention has been directed toward the use of DCs in the treatment of cancer. Our focus on immunotherapy involves the use of agonistic mAb to activate the effector DCs upon NKR-P2 cross-linking. The absolute regulation of NK cell-mediated anti-tumor immune response requires a balance between activating and inhibitory receptors whereas in vitro activation of DCs with fixed tumor cells demonstrates that NKG2D ligand on tumor cells unconditionally activates NKG2D/NKR-P2-harboring DCs. Ligand⁺ tumor cells create a hostile tumor microenvironment in the proximity of DCs. The similar hostile conditions could exist in vivo, when DCs migrate toward the tumor site. Usually immature DCs are present at the periphery of tumor, and after interaction (receptor-ligand) with tumor, they execute the effector function. Our in vitro co-culture assay (fixed tumor: live DCs) proves the natural access of ligand to DCs that is critical for access of ligand on tumor cells. In vivo being a complex environment, complete access of NKR-P2 ligand could be hindered by various factors. 1A6-mediated activation mimics a condition where easy accessibility of ligand was provided to DCs to mount the enhanced anti-tumor responses in vivo. Previously, we have shown 1A6 mAb inhibiting the NK cytotoxicity against YAC-1 targets (13), pre-treatment of NK cells with 1A6 also blocked cytotoxicity against AK-5 cells. Similarly, the killing of Rae1-expressing CHO cells by NK cells was completely blocked by 1A6 (21). We did not find any activation of NK cells by 1A6 in vivo (Fig. 2C). These observations confirm that 1A6 acts as a blocking antibody for NK cells in vitro and has no effect on NK cells in vivo. We have never observed activation of NK cells by 1A6 in vivo even upon prolonged treatment with higher concentrations of mAb1A6 (Srivastava, R. M and Khar, A, unpublished observations). Under normal conditions, murine CD4⁺/CD8⁺ T cells do not express NKG2D receptor. These observations suggest that neither NK cell nor CD4⁺/CD8⁺ T cells would participate directly in 1A6-mediated anti-tumor immune responses in vivo. In the present study, we show the efficacy of agonistic anti-NKR-P2 mAb (1A6) on DCs, which mimicks NKR-P2 ligand and successfully generates protective immune responses through DCs. 1A6 is an IgM class of antibody and efficiently cross-link NKR-P2 to evoke physiological responses whereas bivalent polyclonal serum fails to provide stimulatory signal to the receptor. A similar potential of IgM mAb that modulates DC function through B7-DC also confer the efficiency of pentameric monoclonals, which binds its target with high avidity (28).

Since DCs are a heterogeneous population and their functional criteria are dependent on their anatomical and differential maturation status (35), we have shown the presence of NKR-P2 on in vitro generated BMDCs, which generate both lymphoid and myeloid lineage of DCs as well as DCs isolated from peritoneal cavity and spleen. Recently, we have shown the significance of peritoneal antigen-presenting cells in the regulation of s.c. transplanted AK-5 tumor (36). The efficiency of immunotherapy against tumors depends on the migratory properties of the effector immune cells. DCs are well known for its early migration capacity toward the tumor. The slow growth of transplantable tumors in our experiments illustrates the faster action of DCs. Byrne et al. (37) have also shown that DCs rather than MHCI⁺ macrophages are associated with skin tumor regression. Our data related to the activation of PDCs through mAb1A6 provides an insight into the competent mode of resident DC activation over intravenous DC injection since they are rapidly sequestered by lung macrophages and are required in large numbers for immunotherapy. An i.p. mode of DC activation with 1A6 also enhances the activation of SDCs, which otherwise could not be achieved up to similar levels in vitro. However, activation of SDCs with 1A6 substantiates the stable pharmakokinetic nature of 1A6 in the plasma, which is generally endowed with long half-life.

DCs activated with mAb1A6 produce NO. In vitro INOS induction in DCs has been demonstrated with heat shock proteins (38), IFN-γ, endotoxins (39) and upon CD40 ligation (25). TNF/iNOS-producing subset of DCs are also found, which encounter invading pathogens (40). Enhanced NO production is reported in mouse thymic DCs in response to self- and allo-antigens (41). Mouse BMDCs have also been shown to perform tumoricidal action through NO (42). We have also observed NO secretion by mouse DCs upon 1A6 cross-linking (Srivastava, R. M and Khar, A, Unpublished observations). NO is a well-known tumoricidal molecule that executes tumor cell killing by down-regulating cyclin D1, inhibition of vital enzymes essential for tumor growth and
Fig. 7. A) Monoclonal 1A6 induces synthesis of immunoregulatory cytokines. BMDCs and SDCs were incubated with mAb1A6 (50 μg ml⁻¹) or fixed BC-8 cells (25:1 ratio) for 24 h after incubation; total mRNA from DCs was extracted and subjected to RT-PCR with IL-12-, TNF-α-, IFN-γ- and IL-1β-specific primers. GAPDH signals were also amplified from experimental samples to confirm loading controls. Similar amplification pattern was obtained in three independent experiments. Isotype control mAb does not induce up-regulation of cytokine transcripts (data not shown). (B) Agonistic properties and in vivo anti-tumor activity of mAb1A6. Tumor growth and regression kinetics in rats challenged with AK-5 tumor s.c. and co-administered with mAb1A6 either i.p. or s.c. Data are representative of two similar experiments with three animals per group. (C) In vivo analysis of SDCs upon mAb1A6 injection. Double staining of OX-62-positive SDCs with B7-2- and iNOS-specific mAb upon 1A6 injection. SDCs were obtained from spleen of isotype mAb and 1A6-injected rats and from normal rats. Data are representative of three similar experiments.
DC maturation with anti-NKR-P2 antibody

activation is important since NO produced by activated DCs acts as natural sensitizer to tumor and also promotes DC-mediated tumor cell death.

NKR-P2/NKG2D recognizes its ligand and activates the immune cells. Our data show involvement of p38 MAPK, PI3K and ERK1/2 pathways in 1A6-mediated DC activation. Both iNOS induction and maturation markers were significantly down-regulated by inhibiting these pathways. Recently, iNOS has also been shown to regulate DC maturation process by inhibiting caspase-like activity of immature DCs (48). PI3K and ERK1/2 inhibitors and EGTA, H7 and genistein caused significant reduction in NO synthesis in 1A6-stimulated DCs. These observations confirm the involvement of protein tyrosine kinase, PKC and MAPK pathway in NKR-P2-mediated signaling and also prove the comparable efficiency of 1A6 with NKR-P2 ligand on DCs.

NFκB activation plays a pivotal role in iNOS induction and DC maturation, thus governs both innate and adaptive immunity. Cytotoxic NO production after 1A6 treatment represents strong induction of iNOS, which is reported to be regulated by NFκB activation. Furthermore, NFκB acts as a potential transcription factor in the initiation of MHC II, CD86 and CD80 up-regulation, as well as in IL-12 and TNF-α production (49–51). We have observed degradation of IkB-α and translocation of NFκB upon 1A6 cross-linking. NFκB translocation to the nucleus has also been observed upon NKR-P2-mediated stimulation of DCs. Thus NFκB activation confirms it to be the key executor of activation upon NKR-P2 cross-linking.

NKR-P2 is a maturation-associated receptor as its up-regulation has been detected upon DC activation with fixed BC-8 tumor cells, agonistic mAb1A6 and also in DCs obtained from tumor-bearing animals (13, 20). NKR-P2 engagement also enhanced up-regulation of MHC II, CD86 and CD1a, which are involved in antigen-presentation function of DC. 1A6-activated BMDCs and SDCs were also able to cause T cell proliferation in autologous MLR assays. However, SDCs were slightly more efficient which could be due to their maturational status. In another readout, the endocytic capacity of 1A6-stimulated BMDCs and SDCs was significantly inhibited upon 1A6 stimulation, which also suggests it to be a potent maturational stimulus. We have also detected homotypic aggregation of BMDCs and SDCs upon 1A6 stimulation. Cellular aggregation in response to surface antigen signaling is a regulated process and important for cell–cell interaction. Previously, we have shown relocalization of NKR-P2 upon 1A6 cross-linking in SDCs (13), which suggests that DCs form strong cellular interactions with tumor cells during NKR-P2 interaction with its natural ligand on tumor cell surface. Similar clustering events have also been observed upon MHC II and CD43 cross-linking (26, 52) that could drive the formation of ‘DC–T’ cell cluster to provide efficient stimulatory signal.

1A6 treatment also induces DCs to produce higher level of pro-inflammatory cytokines TNF-α, IL-1β, IFN-γ and IL-12. Previously, we have shown enhanced production of IL-12 from DCs with fixed AK-5 cells. IL-12 produced upon DCs activation also augments NK cell cytotoxicity and thus induces NK cell function (13, 20).

Faster regression of AK-5 tumor was observed upon s.c. or i.p. administration of mAb1A6 in tumor growing rats through the activation of caspases (43, 44). 1A6-activated BMDCs and PDCs produce cytotoxic NO in vitro, whereas SDC showed enhanced NO production under in vivo conditions. NO production from SDCs upon administration of 1A6 suggests that SDCs are not resistant to NO-activating signals but do get activated with an unknown molecular signal. The secretion of NO by ex vivo DCs appears to be highly variable (45). The in vivo and ex vivo differences reflect different lineages or activational requirements of DCs. However, in vivo administration of 1A6 activated both peritoneal as well as SDCs, which exerted strong cytostatic action on BC-8 tumor cells in vitro.

We have recently observed strong NO stimulatory capacity of BMDCs upon ligand–NKR-P2 interactions (Srivastava, R. M and Khar, A, unpublished observations). 1A6-mediated NO synthesis by BMDCs, PDCs and SDCs proves the lineage independent universal agonistic nature of the antibody.

We have observed enhanced tumor cell apoptosis after NKR-P2–ligand interaction (Srivastava, R. M and Khar, A, unpublished observations), and in accordance to that 1A6 stimulates DCs to enhance NO-mediated cytostatic and cytotoxic effects on tumor targets as characterized by DNA degradation and Annexin V staining of tumor cells. Recently, NO has also been shown to confer therapeutic activity to DCs and a combinational immunotherapeutic approach has been proposed through DCs after NO-mediated presensitization of tumor (46, 47). In this context, 1A6-mediated DC

Fig. 8. A) 1A6 inhibits tumor growth: effect of 1A6 on rats challenged with AK-5 tumor (i.p., 4 × 10⁷ cells per rat). (B) Prolonged survival of rats challenged with ZAH (i.p., 2 × 10⁷ cells per rat) upon 1A6 co-administration.
suggesting the enhancement of DC-mediated effective immune response. Increased survival of tumor-bearing hosts at low and high dose of tumor demonstrates the capacity of DCs to mount immune responses at highly critical stage. In addition, 1A6-mediated DC activation also generates the protective immune response against growing tumor. Recently, potent anti-tumor immunity has been attained upon intratumoral targeting of DC (53). Previously, we have reported that DCs isolated from 1A6-injected animals exhibit enhanced apoptosis in BC-8 tumor cells in vitro (13). Both iNOS and maturation status of SDCs were enhanced upon 1A6 administration in vivo, which also suggests the possible direct role of DCs in tumor regression in vivo along with other effector cells. Thus, we show that the agonistic mAb1A6 possesses the potential to enhance DC-mediated anti-tumor activity. Recently, an analogous approach of targeting DCs through NKG2D ligand has also been reported, which generates efficient anti-tumor immune response (54).

In summary, our results demonstrate that anti-NKR-P2 mAb-pulsed DCs induce both innate and adaptive immune responses. This study demonstrates specific targeting of the tumor recognition receptor on DCs, which only operates under stress conditions and recognizes multifarious ligands, displaying its efficiency in a broad range. Our results also suggest an important role for NKR-P2 expressed on DCs in stimulating the anti-tumor immune response and the potential use of a mAb against NKR-P2 in cancer immunotherapy.

**Supplementary data**

Supplementary figure is available at [International Immunology Online](https://academic.oup.com/intimm/article/19/5/591/666534). Supplementary table 1 is available at [https://academic.oup.com/intimm/article/19/5/591/666534](https://academic.oup.com/intimm/article/19/5/591/666534).

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**Abbreviations**

BMDC = bone marrow-derived dendritic cell  
DC = dendritic cell  
GM-CSF = granulocyte macrophage colony-stimulating factor  
[^{3}H]TdR =[^{3}H]thyridine  
i.p. = intra-peritoneal  
MLR = mixed lymphocyte reaction  
NFκB = nuclear factor kappa B  
NO = nitric oxide  
PDC = peritoneal dendritic cell  
PKC = protein kinase C  
RT = reverse transcription  
S.DC = splenic dendritic cell  
TNF-α = tumor necrosis factor-α

**References**


DC maturation with anti-NKR-P2 antibody


