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Anti-cancer mechanisms in two murine bone marrow derived-DC subsets activated with Toll-like receptor 4 agonists

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Abstract

Dendritic cells are well-known by their functions in orchestrating both innate and adaptive arms of immune defense. However, under certain conditions, dendritic cells can exert tumoricidal activity. We have elucidated the mechanism of tumor suppression by Toll-like receptor 4 (TLR4)-activated bone marrow-derived dendritic cells (BMDC) isolated from BALB/c mice. We identified that two distinct subsets of BMDC ($CD11b^+CD11c^+$I-A/E^{Int} and $CD11b^+CD11c^+$I-A/E^{high}) have different cytotoxic mechanisms of action. The cytotoxicity of the former subset is mediated through nitric oxide and reactive oxygen species (NO/ROS) and type I interferon (IFN-β), while the latter subset acts only through IFN-β. TLR4 agonists, lipopolysaccharide (LPS) or pharmaceutical grade Immunomax®, activate CD11c-positive BMDC which in turn directly kill 4T1 mouse breast cancer cells or inhibit their proliferation in a MHC-independent manner. These data not only define two populations of BMDC with different mechanisms of direct cytotoxicity, but also suggest that the I-A/E^{int} subset could be less susceptible to counteracting mechanisms in the tumor microenvironment and support investigation of similar subsets in human DC.

Keywords

DC; TLR4 agonist; Immunomax®; mechanism of tumor killing

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Introduction

Anti-neoplastic immunotherapy has had a resurgence of interest due in part to the broad efficacy shown by immune checkpoint inhibitors, CAR-T cells, other cellular therapies, as well as therapeutic cancer vaccine approaches (1). However, the capacity to induce long-term remissions or tumor control for the majority of patients remains elusive. Additional anti-tumor immunotherapeutic strategies will be needed to increase the proportion of patients who achieve long-term benefit from the approach of harnessing their own immune system to kill their cancer cells (2, 3).

Previously, we reported that in the aggressive 4T1 murine metastatic breast cancer model, a pharmaceutical grade immunostimulator, Immunomax® (IMM), acting through TLR4, successfully cured 30% of mice having otherwise lethal metastatic breast cancer (4). IMM-stimulated dendritic cells (DC) activated NK cellular proliferation and anti-cancer cytotoxic activity (4). Engagement and activation of the immune system through TLR4 has been a long-standing objective, typically attempted through the administration of lipopolysaccharide (LPS) or it's analogues, but has proven to not be clinically feasible (5–7). Other TLR4 agonists have not proven to be particularly efficacious and/or safe (8–11). Based on previously published data showing that bone marrow derived dendritic cells (BMDC) activated with the TLR4 agonist LPS acquire killing capabilities toward tumor cells (12, 13), we tested the ability of mouse BMDC in vitro activated with IMM to directly kill 4T1 breast cancer cells as well as elucidated the mechanism of action.

DC are well known as a phagocytic cell type, characterized by stellate morphology, having $CD11c^+$ $MHC\text{-}II^+$ phenotype, abundant in epithelial border tissues and capable of migrating to lymphoid organs, where they present antigens to and stimulate T cells, thus initiating adaptive immune responses against pathogens or cancers (14). In addition to well-known functions in orchestrating both innate and adaptive arms of immune defense it has also been found out that DC under certain conditions can acquire cytotoxic or tumoricidal activity (12, 13, 15–20). Although the direct anti-cancer activity of "killer DC" has been reported in many publications, the molecular mechanisms employed by these cells remain controversial (12, 17, 20–27). TNF-superfamily members, such as TNF-α, lymphotoxin-α/β, Fas-L and TRAIL were considered to be responsible for DC cytotoxicity against different cancer cell lines (20–25). Other studies showed that DC killed different cancers using peroxynitrite, derived from the chemical reaction between NO and reactive oxygen species (ROS) (12, 17, 26). Also, it has been shown that DC inhibit certain cancers by involvement of type I interferons that possess well-documented anti-cancer activities which is being used clinically (27–30).

Here we report that two distinct subpopulations of TLR4-activated effector BMDC, which can be characterized according to their cell surface markers, have different anti-cancer mechanisms of action.

Materials and Methods

Mice

Female 8- to 10-wk-old BALB/c or C57BL/6J mice were purchased from The Jackson Laboratory or were from the breeder population (Stolbovaya, Russia). All animals were housed in a temperature- and light cycle-controlled facility, and their care was under the guidelines of the National Institutes of Health and the approved Institutional Animal Care and Use Committee protocol at the University of California, Irvine or under standard animal house conditions in the vivarium of the National Research Center Institute of Immunology FMBA. Transgenic mice - CByJ.129P2(B6)-Nos2tm1Lau/J (Nos2 KO), B6.129S-Tnftm1Gkl/J (TNFa KO), B6.129X1-Nox1tm1Kkr/J (Nox1 KO), B6.B10ScN-Tlr4lps-del/JthJ (TLR4 deletion) were purchased from Jackson Laboratory.

Reagents and antibodies

The following reagents were procured as described: CD11c-FITC, CD11c-PE, CD11b-APC, I MHC class II (I-Ad/I-Ed)-FITC, CD40-V421 (BD Bioscience, USA), CD80-FITC, CD86-PE, CD206-PE, CD14-PE, CD115-PE, F4/80-APC, CD103-PE, CD8a-V430, CD197-PE, B220-PE, CD207-PE, DEC-205-PE, CD124-PE, sigH-PE (eBioscience, USA). Blocking antibodies against TRAIL, FasL, TNFR1 (Biolegend, USA), ultrapure mouse rIFNβ, rTNFα (Biolegend, USA). DAPI, Cell Trace Violet (Invitrogen, USA), L-NMMA, β-Mercaptoethanol (Sigma, USA), FeTPPS (Calbiochem, USA), Bx795 (Invitrogen, USA). LPS 055:B5 (TLR tested, Invivogen, USA). LTA-BS, Pam2CSK4, Pam3CSK4, Poly(I:C) HMW, Flagellin-PA Ultrapure, Imiquimod, ODN CpG 1826 (Invivogen, USA). Pharmaceutical grade *Immunomax®* (IMM) has been purchased from Immapharma Ltd (Russia).

Cell lines and culture conditions

Unmodified 4T1 mammary carcinoma cells were obtained from Dr. F.R. Miller (WSU, School of Medicine, Detroit, MI) and were cultured in a complete medium based on DMEM with 25 mM HEPES supplemented with a cocktail of nonessential amino acids, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 10 μg/ml gentamycin (all reagents obtained from PanEco, Russia or Gibco, USA) at 37°C in a 5% CO_2 humidified atmosphere. All cell cultures were maintained under the same conditions. 4T1-GFP stable cell line was obtained by a transfection of 4T1 cells with the lentiviral vector pLV-neo-EGFP followed by FACS-sorting. The E0771 cell line was purchased from CH3 Biosystems, catalog # 94001.

Isolation of bone marrow cells and generation of GM-CSF induced bone marrow derived dendritic cells

Dendritic cells were obtained *in vitro* by culturing bone marrow cells of mice with GM-CSF. Bone marrow was washed out from the femurs and the tibias, in a sterile manner, erythrocytes removed by osmotic shock, nuclear cells washed twice in PBS (Amresco, E404), followed by cultivation in a complete medium supplemented with 20 ng/ml GM-CSF (Biolegend, USA) for 7 days (media refreshed at day 4).

Tumor cell suppression assay in vitro

Tumor cells (either 4T1, 4T1-GFP or E0771) were seeded in the wells of 96-well plate at a density of 500 cells/well in a complete DMEM and cultured in quadruplicates either alone or in the presence of BMDC (at the indicated effector:target ratio, or 50:1 if not shown) in the presence or absence of LPS or IMM (at indicated concentrations, or at 10 μg/ml if not shown). Cell cultures were incubated for 5 days at 37°C and 5% CO_2. On the day of analysis tumor-colonies were either fixed in 2% paraformaldehyde (Sigma) or harvested for flow cytometry analysis with Trypsin solution. Fixed cells were stained with 0.5% methylene blue in 50% ethanol. The area and color density of cancer cell colonies (the integrated color density) were calculated by ELISPOT reader ImmunoSpot (ImmunoSpot, USA).

For FACS analysis (BD FACS Aria II) harvested cells were stained with DAPI and the exact number of tumor cell per well were counted using counting beads as described by the manufacturer (Invitrogen). 4T1-GFP cells were gated as GFP fluorescent, DAPI negative cells. For representation purposes, cell numbers were normalized to the appropriate control value.

Suppression of tumor cells through transwell or by BMDC conditioned media

BMDC conditioned media were obtained from the same culture conditions as described above but without addition of tumor cells. Next day (19 h) culture media from activated or non-activated BMDC cells was collected and centrifuged 10 min at 16000 rpm to exclude cellular contamination, with the supernatant collected before being transferred to the 4T1-GFP cell culture. The supernatants were not subject to freeze thaw before use.

For transwell chamber experiments (0.4uM pore size, SPL Life Sciences), 4T1-GFP cells were seeded into 24-well plate at a density of 1500 cells/well in complete DMEM. BMDC were added to the upper chamber or to the 4T1-GFP cells in the bottom chamber at a 50:1 effector: target ratio, and activated with LPS or IMM (10μg/ml). Five days later the numbers of 4T1-GFP cells per well were quantified with FACS analysis as described above.

Proliferation of 4T1 cells

4T1 cells were stained with Cell Trace Violet kit (Invitrogen, USA) according to the manufacturer's protocol prior to incubation with TLR4 activated or non-activated BMDC as described above. Cell trace Violet distribution (which is proportional to the cells division number) among 4T1-GFP cells was measured by flow cytometry.

Apoptosis of 4T1 cells

4T1 cells were trypsin harvested after 24 and 96 h of coculture with BMDC and analyzed by flow cytometry using FITC Annexin V Apoptosis Detection Kit (BD Bioscience, USA) according to the manufacturer's instructions. Additionally, cells were stained with PI (Invitrogen, USA). 4T1 cells were also stained with Cell Trace Violet before culturing with BMDC for detection of $Annexin^+$ 4T1 cells in mixed culture.

Inhibition of BMDC cytotoxic activity

Target inhibitor [L-NMMA, FeTPPS, β-Me, blocking antibodies (10μg/ml)] were added at indicated concentrations to the BMDC-4T1 cell culture. One hour later BMDC were activated with LPS or IMM and the assays conducted thereafter as described above.

Exosome isolation

BMDC derived exosomes were isolated from conditioned media. BMDC cells were seeded at petri dish at concentration 10^6/ml and stimulated with 10μg/ml IMM or left non-stimulated. 24h later conditioned media was cleared from cells and debris with several steps of centrifugation at 4000g for 30 min at 4°C. Exosomes were then isolated with ultracentrifugation at 100000g for 90 min at 4°C.

Quantification of Nitrites and IFN-β

Nitrites and IFN-β were measured in the conditioned media from the conditions as noted, collected before cell fixation or cell harvesting with trypsin. Nitrites were quantified with Griess Reagent Kit (Invitrogen, USA). IFN-β were quantified with LEGEND MAX™ Mouse IFN beta ELISA Kit (Biolegend, USA) according to the manufacturer's instructions. IFN-β mRNA were analyzed by real-time PCR (forward primer: ACCACAGCCCTCTCCATC; reverse primer: GCATCTTCTCCGTCATCTCC; probe: FAM-CAACCTCACCTACAGGGCGGAC-RTQ). BMDC in additional wells were activated for 2 h with LPS or IMM, then lysed in trizol. mRNA was isolated from each culture and IFN-β mRNA level was determined by normalizing to β-Actin mRNA (forward prmer: AGAGGGAAATCGTGCGTGAC; reverse primer: CAATAGTGATGACCTGGCCGT; probe: FAM-CACTGCCGCATCCTCTTCCTCCC-RTQ).

Dendritic cell sorting

Dendritic cells were sorted from population of GM-CSF induced cells as $CD11c^+$, $I\text{-}A/E^+$ cells (80% from population). All sorting procedures were performed using BD FACSAria™ II Cell Sorter (BD Biosciences). Purity of sorted cells were > 90-95%, dead cells were excluded with DAPI staining. Subpopulations of dendritic cells were sorted with three antibodies; $CD11c^+$ $CD11b^{high}$ $I\text{-}A/E^{int}$ (Macrophage like) or $CD11c^+$ $CD11b^{int}$ $I\text{-}A/E^{high}$ (Dendritic cell like).

Phenotypic analysis of dendritic cells populations

Sorted populations of BMDC cells were stained with target antibodies (see materials and reagents) accompanied with appropriate isotype controls. Thirty min later cells were washed with PBS and fluorescent intensity was analyzed by FACS.

Activated BMDC subpopulations cells were seeded in 96-well plate and activated overnight with LPS or IMM (5μg/ml) or left non-activated. The following day cells were harvested by pipetting, stained with target antibodies, and 30 min later cells were washed with PBS and fluorescent intensity was analyzed by FACS.

Preparation of human dendritic cells and human tumor cell suppression assay *in vitro*

moDC were obtained from blood monocytes in the presence of GM-CSF and IL-4. Briefly, monocytes were purified from freshly isolated human PBMC by EasySep™ Human Monocyte Enrichment Kit (STEM CELL) Magnetic Beads, by negative selection as recommended by manufacturer. Monocytes (5×10^5cell/ml) were cultured in the presence of GM-CSF (50 ng/ml) and IL-4 (50 ng/ml) for 7 days. *In vitro* A549-GFP or K562-GFP cell suppression assay was performed as described in Materials and Methods for 4T1 cells and analyzed by flow cytometry.

Statistical analyses

All statistical parameters were calculated using Graph-Pad Prism 5.0 Software. Statistical significance between groups was determined by two-tailed unpaired t test (P values less than 0.05 were considered significant) and one-way Anova post Tukey comparison test.

For phenotypical analysis cells were stained with antibodies in triplicates and the comparison in surface markers within one cell type before and after TLR4 stimulation was done by Mann-Whitney test and presented on histograms if significant differences were observed ($p<0.05$).

Results

TLR4-activated BMDC are capable of strong inhibition of 4T1 breast cancer cells *in vitro*

To study the anti-cancer efficacy of BMDC activated by the TLR4 agonists IMM and LPS, we used the 4T1 mouse breast cancer cell line and its GFP-expressing derivative, 4T1-GFP cells. The latter was important for the direct detection of cells in appropriate assays requiring the tracking of live 4T1 cells in co-culture studies described below. Notably, both 4T1 and 4T1-GFP mouse breast cancer cells grow and proliferate equally well *in vitro.* Adding TLR4 agonists or co-culturing with mouse BMDC did not change viability and growth characteristics of 4T1 and 4T1-GFP cells (Fig. 1A–F & Fig. S1 A, B). Importantly, co-cultivation of 4T1 or 4T1-GFP cells with BMDC in the presence of IMM or LPS leads to equally strong inhibition of 4T1 and 4T1-GFP viability and growth (Fig. 1 A–F).

We used two complementary methods to quantify cancer cells number and viability. The first is widely used technique for estimation of adhesive tumor cell growth and numbers using methylene-blue staining of tumor cells colonies (Fig. 1C). In this way, colonies of cancer cells are stained and the area and color density of each colony are calculated. The second is a direct measurement of viable 4T1-GFP tumor cell numbers in the co-culture with BMDC using flow cytometry as GFP+/DAPI+ cells (Fig. 1 D, E, F). Both methods showed complementary results.

The inhibitory activity of BMDC was dependent upon both the BMDC:4T1-GFP ratio and the concentration of TLR4 agonists in co-culture (Fig. 1G–J). Specifically, starting at BMDC:4T1-GFP ratio 25:1 (Fig. 1G, H) and at 0.1μg/ml concentration of IMM and LPS (Fig. 1I, J) we observed significant inhibition of tumor growth.

We also tested the ability of the activated BMDC to suppress the growth of various cancer cell lines of different haplotypes. As shown in Fig. 2 A, B, the viability of all tested cancer cell lines is suppressed by activated, but not non-activated BMDC in a dose-dependent manner (effector:target ratio).

Human monocytes-derived dendritic cells (moDC) activated with TLR4 agonist were able to suppress growth of both A549-GFP and K562-GFP cell lines and the cytotoxic effect was dependent on effector:target ratio (Fig 3). Similar data have been obtained with moDC isolated from three other donors (data not shown). There was only slight donor to donor differences in activity of moDC, the maximum cytotoxic effect was measured starting at 0.5 μg/ml of TLR4 agonist concentration.

To understand the basis of this inhibition we measured the percentage of apoptotic 4T1 cells ($Annexin^+$ cells) in co-cultures with activated or non-activated BMDC (Fig. 4A, B). In control co-cultures the percentage of apoptotic 4T1 cells was decreased at 48h and remained in a steady state up to 96h, while in co-cultures with IMM or LPS activated BMDC the percentage of apoptotic 4T1 cells were gradually increased reaching to the highest value at 96h. Significantly higher numbers of apoptotic cancer cells were detected in co-cultures with activated vs non-activated BMDC at all time points of measurement. Thus, TLR4-activated BMDC acquired the ability to induce apoptosis in 4T1 cancer cells. In addition, we demonstrated that simultaneously with apoptosis, there is also an inhibition of the proliferation of tumor cells. Flow cytometry analyses of 4T1-GFP cancer cells at different time-points (24, 48, 72 and 96 h) after their co-cultivation with TLR4-activated or non-activated BMDC (Fig. 4C) showed progressive decrease of 4T1-GFP cancer cells number in co-culture with activated BMDC. A significant decrease in the number of tumor cells was observed starting from day 2 and further (Fig. 4C). Hence, at these time points we analyzed the role of TLR4-activated BMDC in inhibition of proliferation of cancer cells using 4T1-GFP cells. A fluorescent dye dilution assay (cell trace violet) demonstrated that the proliferation of 4T1-GFP cells was significantly inhibited ($p<0.05$, Fig. 4D) in co-culture with TLR4-activated BMDC. Of note, in the presence or absence of IMM or LPS, 4T1-GFP cancer cells (mono-culture) proliferated similarly (Fig. 4C–E & Fig. S1C).

Mechanisms by which TLR4 activated BMDC inhibit 4T1 tumor cell growth

Various mechanisms for the killing activity of DC against different cancers have been reported (12, 17, 20–27, 29, 30). We evaluated whether any of these molecular mechanisms were involved in 4T1 breast cancer inhibition and cytotoxicity by TLR4-activated BMDC. Some of these mechanisms generally are considered to require cell:cell contact (e.g., Fas/FasL, TRAIL, Perforin/granzyme). Others are mediated by soluble factors potentially transferable in culture supernatant (TNF superfamily members and exosomes carrying TNF superfamily members). An additional mechanism, mediated by NO/ROS (peroxynitrite-mediated) is not transferable with supernatant because of its instability (half-life ~ 1 sec).

Activated BMDC suppression of 4T1 cell growth does not require cell:cell contact—Using a double-chamber cell cultivation system, we demonstrated that BMDC activated with TLR4 agonists do not require cell-cell contact for inhibition of 4T1-GFP

tumor cell growth. The inhibition of 4T1-GFP cells by TLR4-activated BMDC cultured in separate chambers was equivalent to the inhibition observed in a mixed co-culture (both types of cells, 4T1-GFP and BMDC in the lower chamber) (Fig. 5A). That is, growth and viability of 4T1-GFP cancer cells were strongly inhibited by activated BMDC isolated through 0.4 μm porous membrane from 4T1-GFP cells in a transwell (Fig. 5A). Additionally, our data showed that TLR4-activated BMDC conditioned media inhibited 4T1-GFP cell growth (Fig. 5B), but the inhibition is significantly weaker ($p \leq 0.01$ for IMM and $p \leq 0.04$ for LPS) than that observed in 4T1-GFP/BMDC co-culture or in BMDC separated from 4T1-GFP cells in the transwell, suggesting contributions of both long-lived and short-lived soluble elements. However, using this experimental design we were not able to distinguish short- and long-lived inhibitory compounds in conditioned media of TLR4-activated BMDC. Studies on possible involvement of exosomes in the anti-4T1-GFP effects of the TLR4-activated BMDC showed high anti-4T1-GFP efficacy of exosome-free fraction, but not exosome-containing fraction of the BMDC conditioned media (Fig. 5 C–E).

TNF family ligands are not involved in a cytotoxic activity of BMDC—To determine if specific TNF family member soluble factors were responsible for the anti-4T1 effects of TLR4-activated BMDC, we further examined possible role of TNF-superfamily members, namely TNF-α, TRAIL and Fas-ligand (Fas-L) in these processes. BMDC activated by TLR4-agonists produced large amounts of TNF-α (Fig. S2A), however, 4T1 cancer cells appeared to be insensitive to recombinant TNF-α (Fig. S2B). Indeed, blocking antibodies, specific to TNF-α, TRAIL, Fas-L or TNF-receptor (TNFR) did not influence anti-4T1 activity of conditioned media from cultures of TLR4-activated BMDC (Fig. 5 F). Together these data indicate that anti-4T1 suppressive activity of TLR4-activated BMDC cannot be explained by BMDC-derived TNF-α or the soluble TNF family members noted above.

IFN-β expressed by activated BMDC suppresses tumor growth—As DC produced IFN-β can have anti-neoplastic and cytotoxic activities (27–30), we tested whether the suppressive activity of the conditioned media towards 4T1 cancer cells could be mediated by IFN-β. TLR4-activated BMDC produce significant IFN-β (Fig. S2C) and recombinant IFN-β significantly inhibits 4T1-GFP cell growth *in vitro* (Fig. S2D). In contrast to TNF family members, there are no readily available neutralizing antibodies to IFN-β. Thus, we employed Bx795, an inhibitor of Interferon Response Factor (IRF)-3 transcription factor and type I interferon synthesis. BX795 completely abrogates the growth inhibition of 4T1-GFP cells by TLR4-activated BMDC in co-cultures (Fig. 6A) and by the conditioned media (Fig. 6B). These data support an important role of IFN-β in the mechanisms of anti-cancer effect of TLR4-activated BMDC. However, we recognize that BX795 has pleotropic effects that may not be dependent upon IRF-3 inhibition. Bx795 has been reported to inhibit the TLR ligand-induced NO release in chicken monocytes (31) as well as in RAW264.7 cells (32). We examined whether this was also present in TLR4 ligand activated BMDC. We found that in addition to inhibiting IFN-β synthesis (Fig. S3A), Bx795 also inhibits the production of NO (Fig. S3D). Bx759 does not inhibit production of IL-6 and TNF-α by TLR4-activated BMDC (Fig. S3B, C), indicating that Bx795 does not have specific toxic effects on BMDC. As our previous data supported the involvement of both

long-lived and short-lived effector molecules and these observations, we cannot state with absolute certainty that IFN-β has a direct independent contribution to TLR4-activated BMDC inhibition of 4T1. The suppressive activity of recombinant IFN-β however, suggests that production of this molecule contributes to the 4T1 suppressive activity of TLR4-activated BMDC.

Inhibition of peroxynitrite formation significantly decreases the anti-tumor properties of TLR4-activated BMDC—We evaluated the possible involvement of a peroxynitrite-related mechanism (12, 17, 26) in TLR4-activated BMDC's cytotoxic activity toward 4T1 cancer cells. We analyzed co-cultures of 4T1 cells and BMDC under three conditions; after inhibition of NO-production in activated BMDC with the NO-synthase inhibitor L-NMMA (33), blocking the generation of ROS by increased concentrations of β-mercaptoethanol (β-ME) (34) and destruction of peroxynitrite with FeTPPS (35) (a widely known peroxynitrite decomposition catalyst and a selective peroxynitrite scavenger) (Fig. 6C–F). Data demonstrated that anti-4T1 cancer activity of TLR4-activated BMDC includes mechanisms that involve NO, reactive oxygen species (ROS), and peroxynitrite- a product of reaction between NO and ROS (Fig. 6G–J). 100 μM of L-NMMA, an inhibitor of all known NO-synthases, completely blocks production of NO (Fig. 6D) and reduces to half the anti-cancer effect of TLR4-activated BMDC in their co-culture with 4T1-GFP cells (Fig. 6G). Peroxynitrite is a short-lived product of reaction between NO and ROS. The activity of FeTPPS, a peroxynitrite decomposition catalyst/scavenger, which reduces to one third the anti-cancer effect of TLR4-activated BMDC in their co-culture with 4T1-GFP cells (Fig. 6H) further supports the involvement of this pathway. β-ME, a reducing agent and antioxidant, also significantly reduces the anti-cancer effect of TLR4-activated BMDC in their co-culture with 4T1-GFP cells (Fig. 6I), albeit at the highest concentration tested. Importantly, unlike in the cell:cell system, the NO-synthase inhibitor (L-NMMA) and peroxynitrite decomposition catalyst (FeTPPS) do not abrogate the anti-4T1-GFP activity of TLR4-stimulated BMDC conditioned media (Fig. 6J), consistent with the short half-life of peroxynitrite (T½ ≈ 1s).

TLR4 activated BMDC from *iNOS* knockout mice retain only partial inhibitory effect on tumor cell growth

To further examine the mechanism of inhibition of tumor growth by TLR4-activated BMDC, we tested BMDC isolated from mice with different genetic knockouts (KO). Most of the commercially available KO mice have a H-2^b background, therefore, although we previously showed that inhibition of tumor growth is not restricted to MHC class II, in these experiments along with 4T1 cell line we also used the E0771 H-2^b murine breast cancer cell line. We demonstrated that KO of *TNF*α and *Nox1* (NADHP$^{-/-}$) did not affect the production of nitrites (and consequently peroxynitrites) and IFN-β in TLR4 activated BMDC (Fig. 7 A). In contrast, BMDC generated from *iNOS* KO animals did not produce nitrites, but did produce high levels of IFN-β (Fig. 7 A) in response to TLR4 activation. TLR4 activated BMDC from TNFα and Nox1 KO mice possess the same inhibitory ability as BMDC from wild type mice, whereas BMDC from *iNOS* KO animals demonstrated nearly half of the inhibitory potential compared with BMDC from wild type mice (Fig. 7 B, C). The fact that there was TLR4 activated BMDC suppressive activity that could not be abrogated by KO of

iNOS, provides additional support for an IFN-β contribution that is independent of NO/ROS and peroxynitrite. BMDC from *TLR4* KO mice failed to respond to LPS or IMM, did not produce either nitrites or IFN-β and did not show any suppressive ability (Fig. 7), consistent with the described TLR4 agonist activity of LPS and IMM.

The combined results of the experiments reported above show that TLR4-activated BMDC use at least two distinct anti-cancer mechanisms for killing cancer cells or inhibiting their growth (Fig. S3 E, F). Namely, these are ROS/NO-derived peroxynitrite and interferon-β mediated mechanisms. Peroxynitrite has a very short life-time and therefore acts only in co-culture of TLR4-activated BMDC and cancer cells. In contrast, Interferon-β is a stable, soluble compound transferable with culture supernatant that contributes independently to the TLR4-activated BMDC suppressive activity.

BMDC activated by TLR3, TLR4 and TLR9 produce peroxynitrites or IFN-β and kill tumor cells

We tested a panel of TLR agonists in the context of ability to stimulate cytotoxic activity of BMDC against 4T1 cell line. In total, we tested 10 ligands of TLR1/2, TLR2/6, TLR3, TLR4, TLR5, TLR7, TLR9 receptors within the wide range of ligand concentration (from 10 pg/ml to 10 μg/ml). Data indicated that TLR7 ligand was toxic for the 4T1 cells, while other TLR ligands can or cannot induce tumoricidal activation of BMDC (Fig. 8A). More specifically TLR1/2, TLR2/6, TLR2 and TLR5 ligands did not induce BMDC cytotoxicity against 4T1 tumor cell line (Fig 8A) as well as production of either peroxynitrites or IFN-β (Fig. 8 B, C). Importantly, TLR3, TLR4 and TLR9 ligands did induce similar cytotoxic activity of BMDC (Fig. 8 A) and these activated cells produce peroxynitrites or IFN-β (Fig. 8 B, C). Of note, all agonists have been used at the concentrations that strongly activate BMDC to produce TNF-α (Fig. 8D).

Two different subpopulations of BMDC are responsible for inhibition of cancer cell growth via peroxynitrite/interferon-β or interferon-β alone

BMDC described above were generated in the standard fashion from mouse BM cells enriched with $CD11c^+$ $I\text{-}A/E^+$ cell population and cultured with GM-CSF. We found that highly purified TLR4-activated $CD11c^+I\text{-}A/E^+$ BMDC fully inhibit 4T1 cancer cells growth as compared to that observed with unsorted BMDC (Fig. S4). However, recently it has been shown that the $CD11c^+I\text{-}A/E^+$ population of conventional BMDC is heterogenous with two subsets distinguishable by the level of expression of MHC class II molecules (MHC-II) on their surface (36). One of these BMDC-subtypes expresses high ($CD11c^+CD11b^+MHC\text{-}II^{high}$) and the other intermediate or low ($CD11c^+CD11b^+MHC\text{-}II^{Int}$) MHC-II molecules.

We hypothesized that the two distinct mechanisms of anti-4T1 activity shown in this study may be associated with these two different subtypes of BMDC. Hence, we purified $CD11c^+CD11b^+I\text{-}A/E^{high}$ and $CD11c^+CD11b^+I\text{-}A/E^{Int}$ subpopulations of BMDC, and studied the production of NO and interferon-β by these cells as well as the acquisition of the ability of these cells to kill 4T1-GFP cells after activation with TLR4 agonists (Fig. 9). In our hands, conventional GM-CSF-differentiated BMDC were mostly (~60%) composed of $CD11c^+CD11b^+I\text{-}A/E^{Int}$ and at a lesser extent (~30%) of $CD11c^+CD11b^+I\text{-}A/E^{high}$ (Fig. 9A). Upon

activation with TLR4-agonists, sorted $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$ population produced nitrite (Fig. 9F), while sorted $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$ did not produce detectable nitrite (Fig. 9G). Both subsets of TLR4-activated BMDC populations produced interferon-β (Fig. 9F, G). Consistent with these findings, the conditioned media from both of the BMDC subsets were capable of inhibiting the growth of 4T1-GFP cells (Fig. 9I, J). However, in the co-culture setting, TLR4-activated $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$ cells producing both NO/peroxynitrite and INF-β had a more pronounced effect on 4T1-GFP cancer cells (Fig. 9I) as compared to $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$ cells (Fig. 9J) supporting that both mechanisms were available and used by the $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$ DC subset. Thus, two anti-4T1 cancer mechanisms are unequally shared between two distinct subpopulations of BMDC. TLR4-activated $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$ cells generate both NO/peroxynitrite and interferon-β, while $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$ produce only interferon-β, but not NO/peroxynitrite (Fig. 9F, G).

Differential expression of surface markers in the two subpopulations of BMDC

To further characterize these two subsets of BMDC we evaluated a panel of antibodies specific to cell surface markers for macrophage and DC lineages. We showed that $I\text{-}A/E^{Int}$ population exhibits a more macrophage-like phenotype with increased expression of CD115 (MCSF receptor), CD206 (mannose receptor), CD103 and F4/80 (Fig. 10B). The $I\text{-}A/E^{high}$ population lack CD206 or CD115 expression, but had increased DEC205 and B220 surface markers (Fig. 10B). Surface expression of molecules associated with co-stimulation, such as CD80, CD86, CD40 was significantly higher in the $I\text{-}A/E^{high}$ cells compared with $I\text{-}A/E^{Int}$ cells and upon LPS or IMM stimulation the expression of these molecules in $I\text{-}A/E^{Int}$ cells increased, reaching the level in $I\text{-}A/E^{high}$ cells (Fig. 10 C, D). The I-A/E expression levels were not changed significantly in both populations of sorted cells after TLR4 activation. Confocal microscopy revealed different morphologies for these two subsets. Cells in the $I\text{-}A/E^{high}$ population possess high numbers of dendrites (phylapodia), whereas the majority of $I\text{-}A/E^{Int}$ cells were presented as big macrophage-like cells (Fig. 10 A).

Discussion

In this study, we demonstrated that a pharmaceutical grade TLR4 agonist, IMM, as well as the classical TLR4 ligand LPS, induced anti-proliferative and cytotoxic activity of BMDC against 4T1 cancer cells, and comprehensively investigated the mechanisms involved in the killing activity of BMDC. We showed that TLR4-activated BMDC have two principal effects on 4T1 cells; inhibition of proliferation of 4T1-GFP cells and induction of apoptosis and secondary necrotic 4T1 cell death (37). Both IMM and LPS showed comparable efficacy in our experiments. However, LPS is not a clinically relevant TLR4 agonist due to toxicity in humans. IMM has been employed in Eastern Europe in a range of patients with different local and systemic infectious diseases. Importantly, we have demonstrated for the first time that IMM/TLR4-activated BMDC use at least two distinct anti-cancer mechanisms for killing cancer cells or inhibiting their growth, a ROS/NO-derived peroxynitrite and an interferon-β mediated mechanisms. We showed that these mechanisms are unequally shared between two distinct subpopulations of BMDC, $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$ and $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$.

Direct anti-cancer activity of DC is not new *per se*, as there is a substantial literature dedicated to the so-called “killer DC” (15, 16). However, there is no consensus in the literature concerning the phenotype of such anti-cancer DC, the panoply of exogenous and endogenous ligands inducing the cancer-killing activity, and molecular mechanisms of cancer-inhibition/cytotoxicity molecular machinery. Direct anti-cancer activity of the following DC subtypes has been reported: (i) conventional DC *in vitro* differentiated from rodent bone marrow cells in the presence of GM-CSF with or without IL-4, (13, 26), (ii) DC purified from rodent spleen, lymph nodes (38, 39) or epidermis (40), and (iii) human DC *in vitro* differentiated in the presence of GM-CSF and IL-4 from monocytes obtained from the peripheral blood of healthy donors or cancer patients (17, 20). Our studies are distinct from those which use murine cells that express characteristics of both DC and NK cells and are termed natural killer dendritic cell (NKDC) or interferon-γ-producing killer DC, which belong to the NK cell but not to DC lineage (41–43).

DC-mediated anti-cancer mechanisms are based on the inhibition of target cell division or direct killing, or both. We demonstrated that cell:cell contact was not necessary for TLR4-activated BMDC anti-4T1 cancer effects and cytotoxicity is mediated by a restricted subset (Interferon-β and NO/ROS/peroxynitrite) of soluble factors produced by DC.

According to the published studies, different pathways could be involved in a target cell killing by DC such as granzyme (44), TNF-superfamily members such as TRAIL, Fas-L and TNF-α (16, 18, 20–25, 45–48). It has been reported that exosomes, bearing TNF-superfamily members, namely, TRAIL, Fas-L and TNF on their surface, are responsible for anti-cancer activity in the supernatant of activated BMDC (25). We do not believe that exosomes are involved in anti-4T1 cancer effects of TLR4-activated BMDCs, since neither discarding the exosomes by ultracentrifugation nor antibody-mediated blocking of TRAIL, Fas-L and TNF-α abrogated or weakened the anti-cancer activity. The differences in target cancer cell (4T1 mouse breast cancer versus B16 melanoma) may account for the differences between our data that of others (25). Although TNF-α significantly suppresses growth of B16 melanoma cell line (49) recombinant TNF-α does not influence *in vitro* 4T1 breast cancer cells growth.

Interferon-β has pleiotropic effects: it is acknowledged to inhibit cancer cell proliferation, is important for DC-presentation of cancer-associated antigens to T cells, and plays a role in T cell intra-tumoral recruitment, as well as prevention or inhibition of metastatic foci growth (27–30). We demonstrated that TLR4-activated BMDC produce abundant interferon-β and hypothesized that it might play a role in the acquired cytotoxic activity of TLR4-activated BMDC.

We showed that recombinant interferon-β suppressed 4T1 cancer cells in a dose dependent manner and inhibition of interferon-β synthesis by compound Bx795 effectively abrogated anti-4T1 cancer activity of TLR4-activated BMDC. We documented that Bx795 is not a highly selective inhibitor and also suppresses production of NO by TLR4-activated BMDC along with inhibition of type I interferon synthesis, without any significant decrease in production of other pro-inflammatory compounds such as IL-6 and TNF-α. Our experiments in INOS KO mice, where long lived soluble factors retained cytotoxic activity in the absence

of NO/ROS/peroxynitrite, support the significant role of IFN-β in anti-4T1 cancer activity of TLR4-activated BMDC.

In addition to IFN-β, we demonstrated that peroxynitrite plays a key role in 4T1-cancer inhibition and killing by TLR4-activated BMDC. Peroxinitrite is known as a short-lived (~1 sec) cytotoxic compound produced as a result of chemical reaction between ROS, such as superoxide anion ($^{\bullet}O_2^-$), and nitric oxide (NO). It is widely accepted that production of ROS and NO are characteristic features of TLR4-activated DC and macrophages. Bonnotte, Larmonier and colleagues, among others, identified the importance of the DC-produced NO- and ROS-dependent peroxynitrite as a molecular mechanism killing cancer target cells (12, 17, 26). In their model system, as in ours, there was no role for members of the TNF-superfamily (12). In our study, we showed that peroxynitrite is responsible for anti-4T1-cancer effects of TLR4-activated BMDC in co-culture with 4T1-GFP cells and inhibition of peroxynitrite production substantially decreased anti-4T1 cancer activity of TLR4-activated BMDC suggesting a key role of peroxynitrite. We confirmed these data using BMDC from *iNOS* KO animals that had less than 50% abrogation of the cytotoxic activity compared with BMDC from wild type mice (Fig. 7 B, C). As noted above, this provides additional support for an IFN-β contribution that is independent of NO/ROS and peroxynitrite.

Additional studies that we are reporting herein reveal that two different anti-cancer mechanisms are differentially associated with two different DC subsets within the population of CD11c-positive BMDC. Using FACS-sorted 91-92% pure cell populations we showed that upon TLR4-activation, $CD11c^+CD11b^+I\text{-}A/E^{Int}$ cells intensively produce NO, while $CD11c^+CD11b^+I\text{-}A/E^{high}$ cells are incapable of NO-production. Both subsets of TLR4-activated BMDC produced interferon-β. Reis e Sousa et al have studied these $CD11c^+CD11b^+I\text{-}A/E^{Int}$ and $CD11c^+CD11b^+I\text{-}A/E^{high}$ subpopulations of conventional BMDC (36) in terms of their exome and phenotype profiles (36), but not their cancer-killing or suppressing activities. To the best of our knowledge, this is the first time that the molecular mechanisms of direct anti-cancer activities of these BMDC subpopulations have been described. According to Helft et al. 2015 (36), $CD11c^+CD11b^+I\text{-}A/E^{high}$ cells represent classical dendritic cells, while $CD11c^+CD11b^+I\text{-}A/E^{Int}$ cells have characteristics of non-classical $CD11c^+$ macrophages. If this is confirmed, our data would suggest that studies using GM-CSF-differentiated conventional BMDC should be re-considered through the prism of newly discovered $CD11c^+$ macrophages hidden within $CD11c^+I\text{-}A^+$ BMDC population. These macrophages are solely responsible for NO/peroxynitrite production and associated anti-cancer activity.

Although, there is a species-specific variation in the expression patterns of TLR4 receptor in general, TLR4 genes are highly conserved across mammalian species. Specifically, humans and mice have similar distribution of TLR4 in cells of myeloid lineage, monocytes, macrophages, dendritic cells and B cells with exception of microglia (50). Not surprisingly, both mouse BMDC and human moDC activated with TLR4 agonists IMM and LPS inhibited the growth of appropriate cancer cell lines (Fig. 2&3). However, to our knowledge, currently it is not known that populations of DC similar to described in this paper exist in humans and if they do, further studies should be conducted to understand if there are any differences in therapeutic potency of these populations. If this is the case, an activation of

appropriate DC population with an ability to more effectively acquire the cytotoxic activity upon stimulation with TLR-agonist can be useful in clinic, for example during a cell-based personalized therapy.

Thus, our findings show that (i) stimulation with two TLR4 agonists, IMM and LPS, can re-program mouse conventional BMDC and human moDC to acquire cancer cell killing/growth inhibiting activity; (ii) two different mechanisms are responsible for this activity, peroxynitrite- and IFN-β; (iii) cell contact is not necessary for killing activity; and (iv) two different mechanisms are associated with distinct subpopulations of BMDC. Based on these findings, and taking into account that IMM has been in clinical use in Eastern Europe as an anti-viral and anti-bacterial drug where it has been shown to be safe and non-toxic, we believe that it could be readily translated into the clinical arena providing an additional component to the anti-neoplastic immunotherapeutic arsenal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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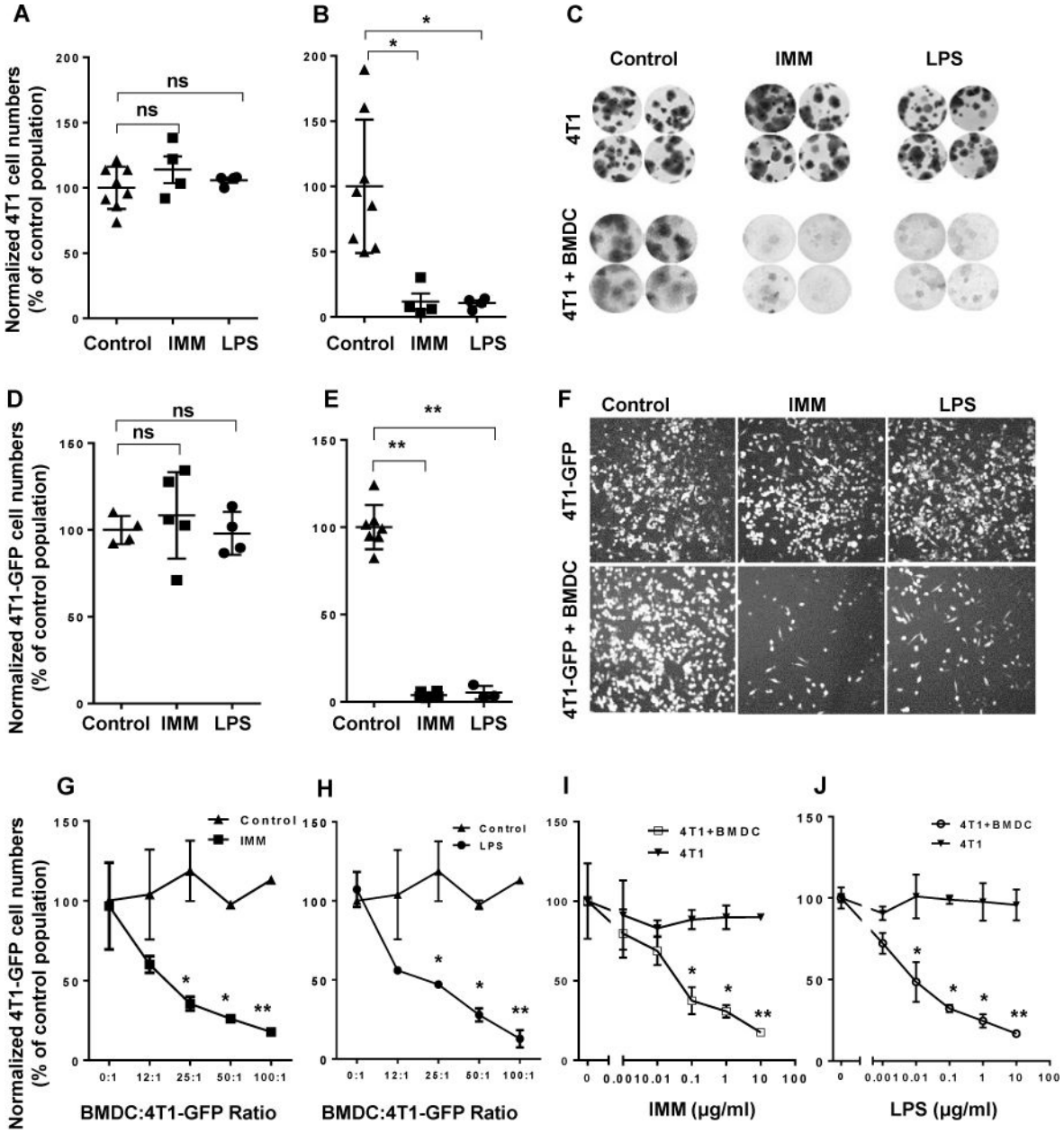

Fig. 1. Conventional BMDC activated by TLR4-agonists, IMM and LPS, strongly inhibit growth of 4T1 or 4T1-GFP mouse breast cancer cells. Killing activity of TLR4-activated BMDC depends on BMDC:4T1-GFP ratio (G,H) and concentration of TLR4 agonists, IMM (I) and LPS (J)

Growth of 4T1 **(A,B,C)** or 4T1-GFP **(D,E,F)** cells in the absence **(A,D)** or presence **(B,E)** of BMDC activated with IMM (5 μg/ml) or control TLR4 agonist, LPS (5μg/ml). Of note, TLR4 agonists do not effect growth of 4T1 **(A)** or 4T1-GFP **(D)** tumor cells. Representative images of wells with colonies of 4T1 cells stained with methylene blue (0.5%) **(C)** and 4T1-GFP cells visualized via fluorescent microscopy **(F)** are presented. (**G,H**) 4T1-GFP cells were co-cultured with BMDC at effector:target ratio from 0:1 to 100:1 without or with IMM (5 μg/ml) (**G**) or LPS (5 μg/ml) (**H**). **(I,J)** 4T1-GFP cells were co-cultured without or with BMDC (E:F-50:1) and different concentrations of IMM (**I**) or LPS (**J**).

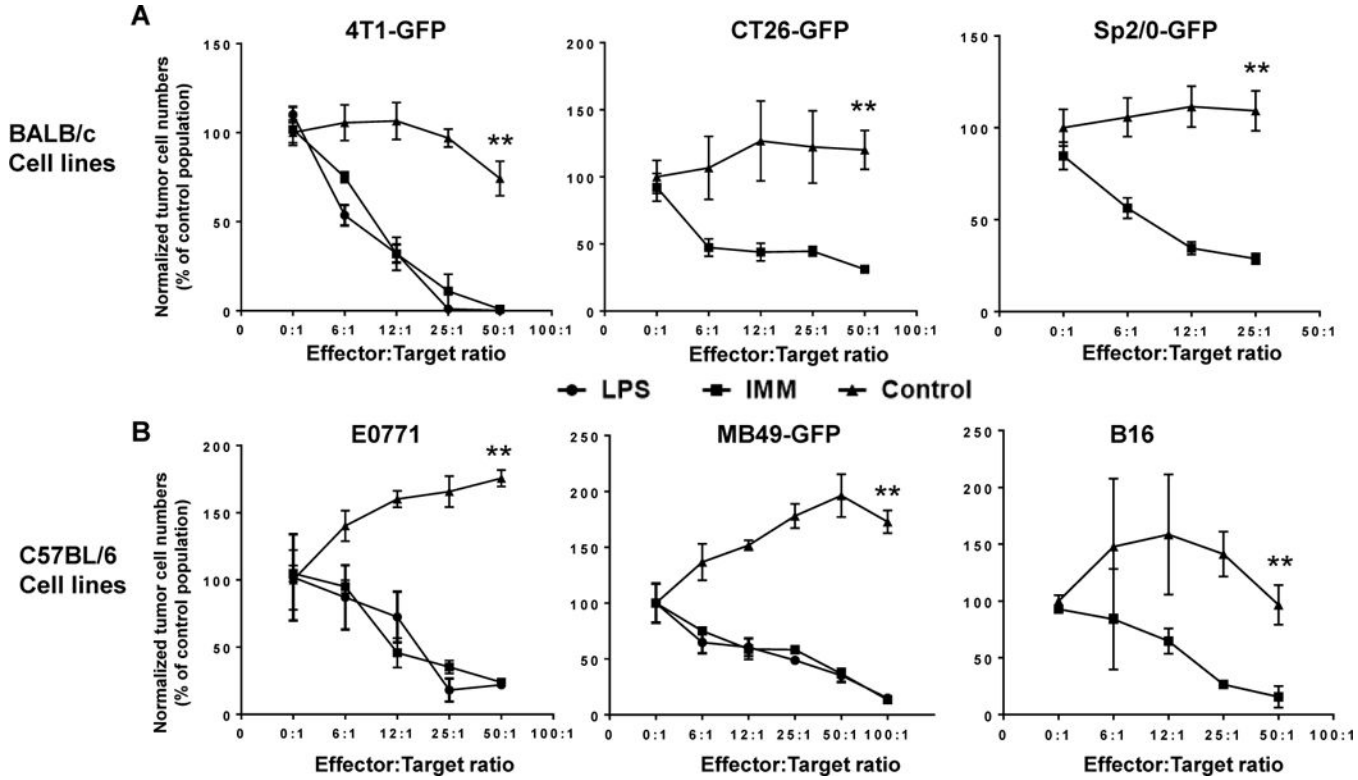

Fig. 2. Conventional BMDC activated by TLR4-agonists, IMM and/or LPS, strongly inhibit growth of indicated mouse cancer cell lines (4T1-GFP and E0771-breast cancer, CT26-GFP-colon cancer, Sp2/0-GFP-hybridoma, BM49-GFP-urothelial carcinoma, B16-melanoma)

Inhibiting activity of TLR4 activated BMDC depends on BMDC:target ratio. Cells of each haplotype was co-cultured with BMDC of the relevant origin.

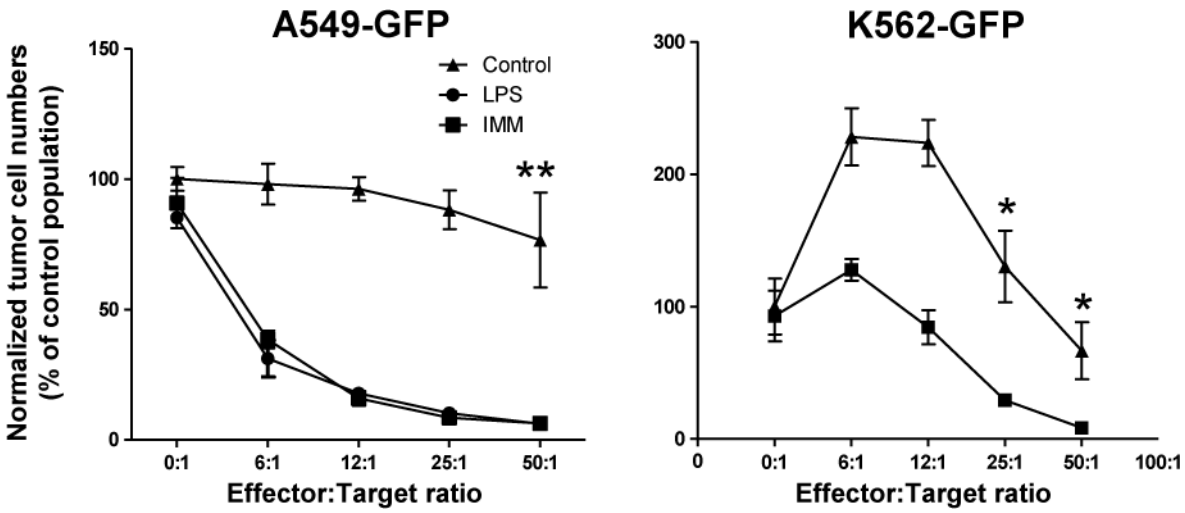

Fig. 3. Monocyte-derived human dendritic cells activated by TLR4-agonists strongly inhibit growth of human cancer cell lines

A549-GFP or K562-GFP cells were co-cultured with moDC isolated from PBMC of healthy donors at effector: target ratio from 0:1 to 50:1 without or with IMM (5 μg/ml) or LPS (5 μg/ml). Similar results have been obtained with three different donors.

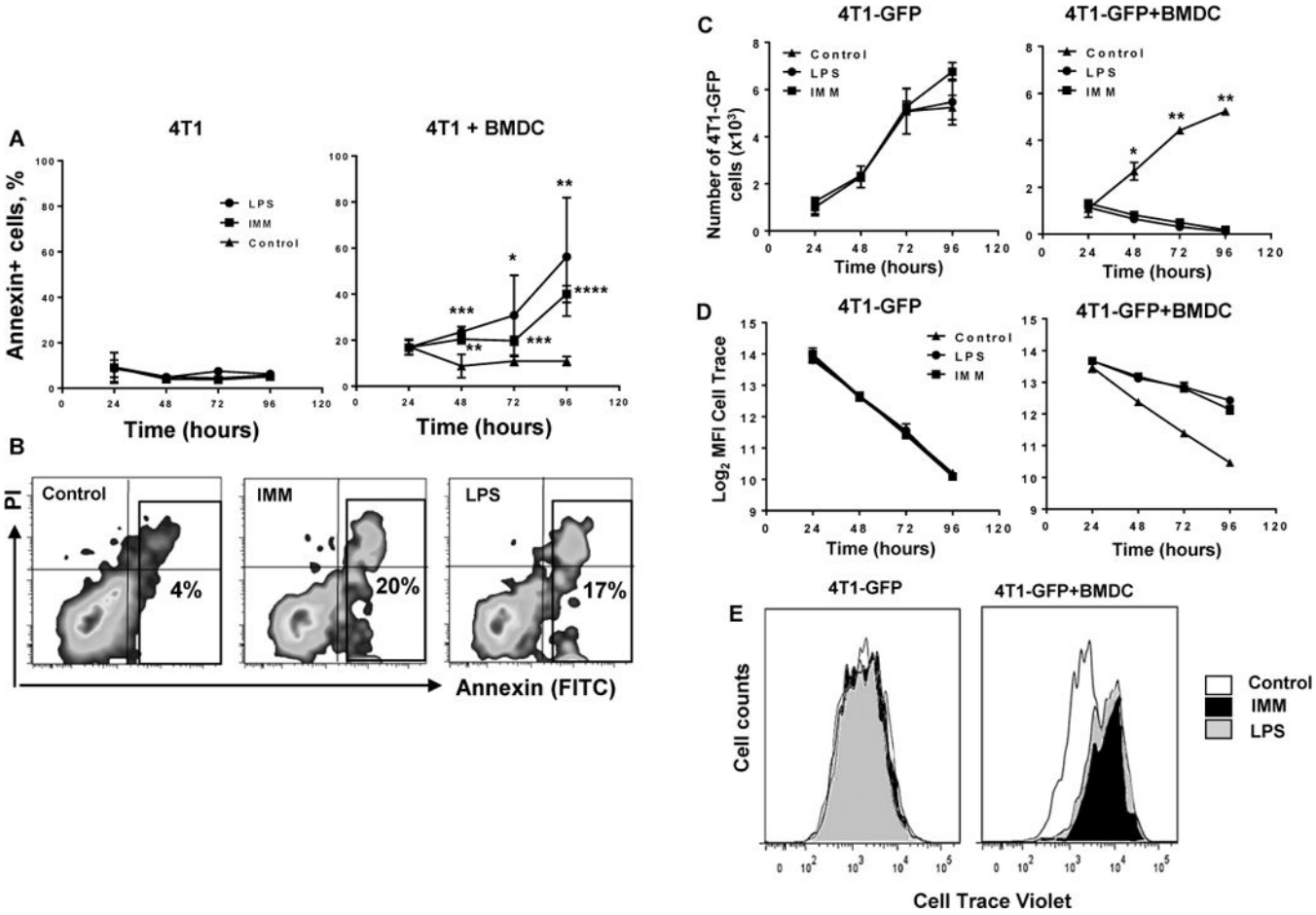

Fig. 4. TLR4-activated BMDC induce apoptotic death and inhibit the proliferation of 4T1-GFP cancer cells in co-culture. (A)

An annexin positive 4T1 cells grown alone or in the co-culture with BMDC with or without IMM (5μg/ml) or LPS (5μg/ml) taken from 24 to 96 hours.

(**B**) Representative analyses of apoptotic 4T1 cells by FACS after 48 hours of co-culturing with IMM or LPS activated or non-activated BMDC.

(**C**) Kinetics of 4T1-GFP grown alone or in the co-culture with BMDC with or without IMM (5μg/ml) or LPS (5μg/ml) taken from 24 to 96 hours.

(**D**) 4T1-GFP cells' division rate detected by Cell Trace Violet fluorescent intensity (Log2) of 4T1-GFP grown alone or in the co-culture with BMDC with or without IMM (5μg/ml) or LPS (5μg/ml) taken from 24 to 96 hours.

(**E**) Representative histograms showing the cancer cell proliferation measured by Cell Trace Violet fluorescent signal in 4T1-GFP cells grown alone or in the presence of BMDC with or without IMM or LPS after 72 hours.

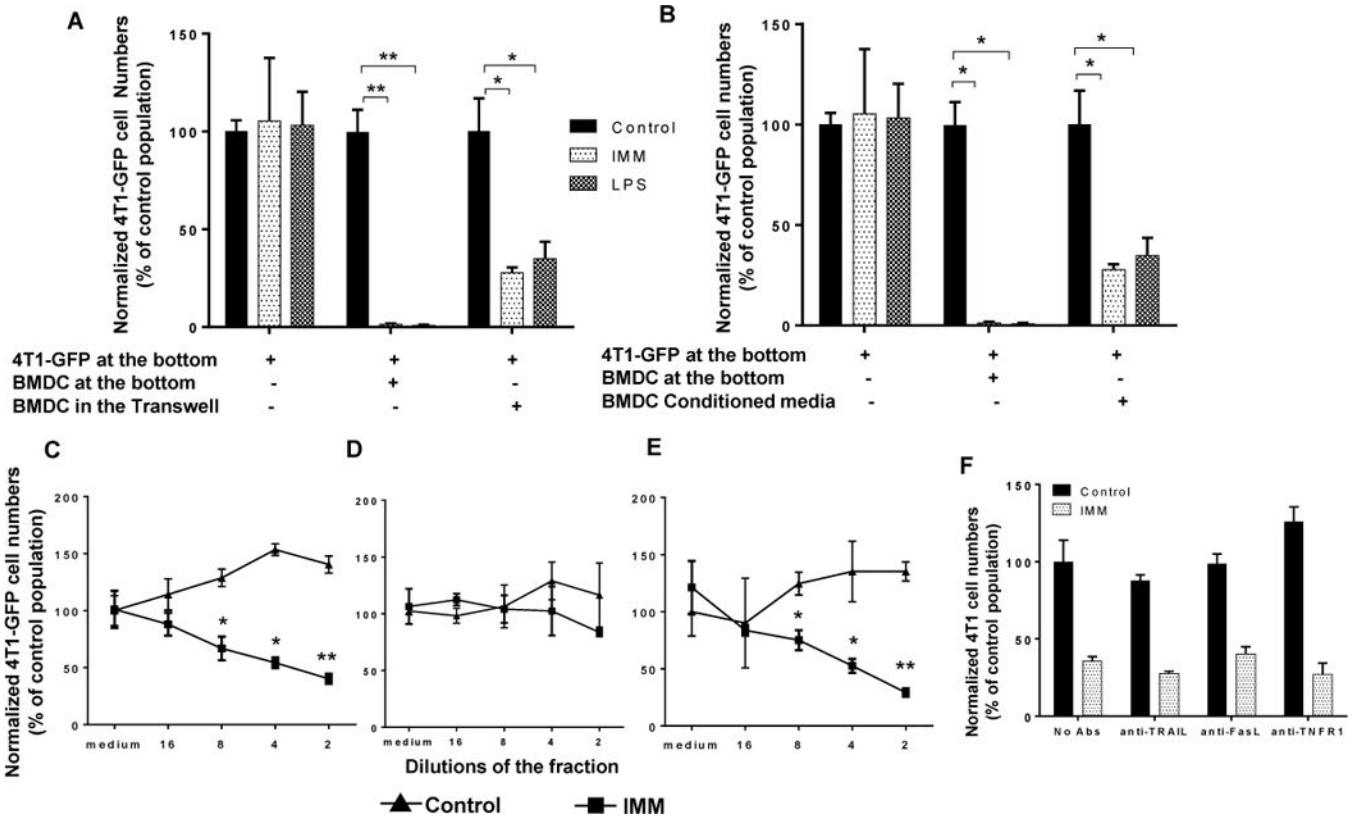

Fig. 5. TLR4 activated BMDC inhibit 4T1-GFP growth distantly (A, B) by soluble factors (C-E), but not mediated by TNF-superfamily members (F). (A)

Inhibition of 4T1-GFP cells by BMDC with or without IMM or LPS in direct co-culture or separated via the Transwell 0.4 μm pore size polycarbonate membrane. **(B)** Inhibition of 4T1-GFP cells grown in the direct co-culture of BMDC or with BMDC conditioned medium after 24 h incubation with or without IMM or LPS. **(C)** Inhibition of 4T1-GFP cell growth by different dilutions of added conditioned medium of BMDC non-activated or activated by IMM. (**D)** Inhibition of 4T1-GFP cells growth by different dilutions of exosomes isolated from conditioned medium of BMDC non-activated or activated by IMM. (**E)** Inhibition of 4T1-GFP growth by different dilutions of exosomes-free fraction of conditioned medium of BMDC non-activated or activated by IMM.

(F) Blocking of suppressive activity of conditioned medium of BMDC non-activated or activated by IMM with antibodies to TNF-family ligands or receptors (TRAIL, FasL, TNFR1).

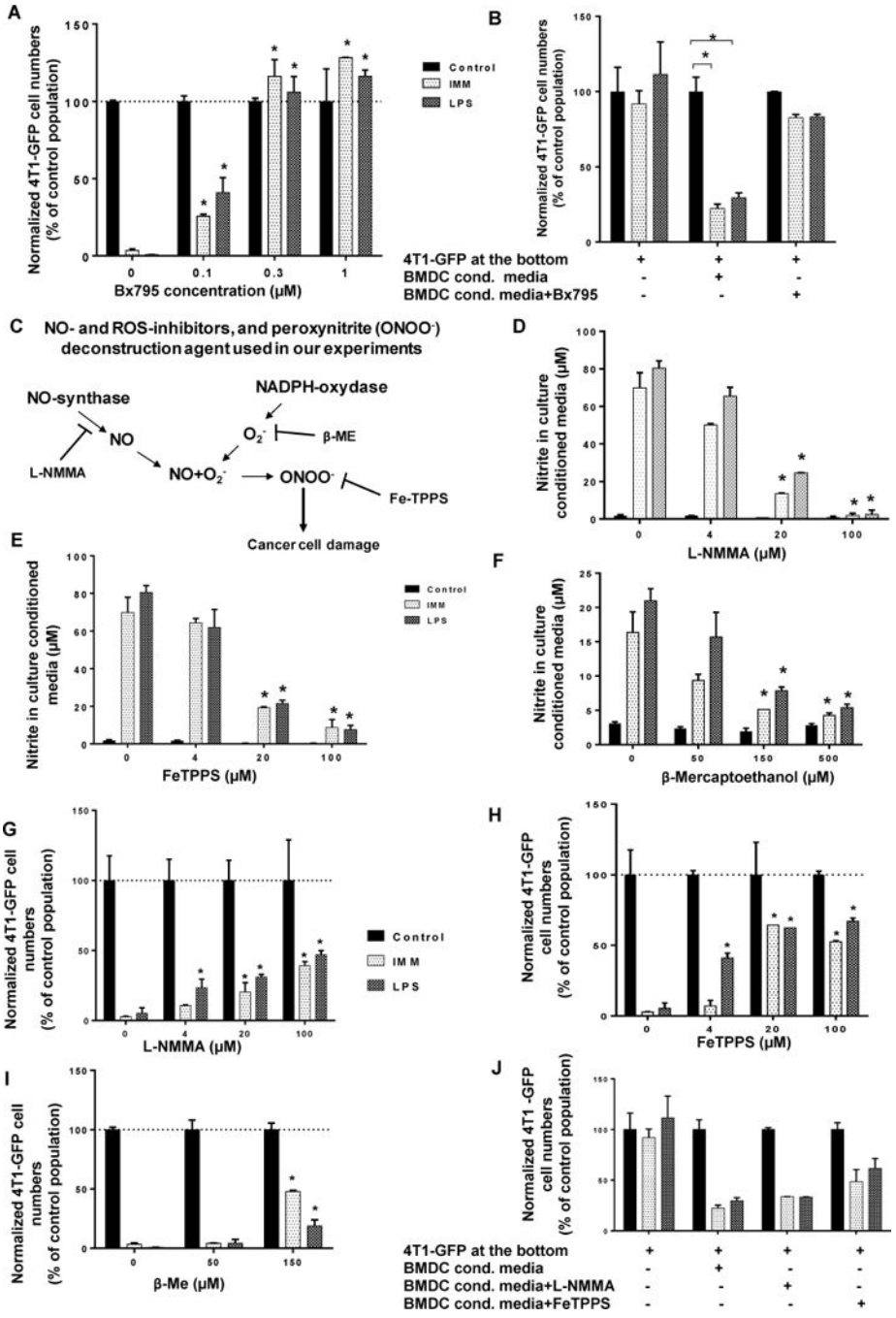

Fig. 6. Inhibition of 4T1-GFP cells growth by TLR4 activated BMDC is partially associated with IFN-β and partially peroxynitrite. (A)

Dose-dependent inhibition of type-I-Interferon production (TBK1 kinase) by Bx795 during the coculture of 4T1-GFP with BMDC activated with IMM or LPS or non-activated. (*Note: asterisks indicate significant difference in comparison to respective 4T1-GFP growth suppression without Bx795). **(B)** Growth inhibition of 4T1-GFP cells by BMDC conditioned medium obtained after 24 h incubation with or without IMM or LPS in the presence or absence of Bx795. **(C)** Schematic diagram depicting inhibition steps in peroxynitrite

pathway with indicated inhibitors. **(D-F)** Quantity of nitrites measured in the non-activated or TLR-4 activated BMDC conditioned media after 24 hours of incubation with different concentrations of L-NMMA **(D)**, FeTPPS **(E)** and β-Mercaptoethanol **(F)**. **(G)** Dose-dependent inhibition of NO-synthase with L-NMMA during the coculture of 4T1-GFP with BMDC activated with IMM or LPS or non-activated. **(H)** Dose-dependent destruction of peroxynitrites with FeTPPS during the coculture of 4T1-GFP with BMDC activated with IMM or LPS or non-activated. **(I)** Reduction of ROS by β-Mercaptoethanol during the coculture of 4T1-GFP with BMDC activated with IMM or LPS or non-activated (* Note: asterisks indicate significant difference in comparison to respective 4T1-GFP growth suppression without inhibitor). **(J)** Growth inhibition of 4T1-GFP cells by BMDC conditioned medium obtained after 24 h incubation with or without IMM or LPS in the presence or absence of indicated inhibitors (L-NMMA 100μM, FeTPPS 50μM).

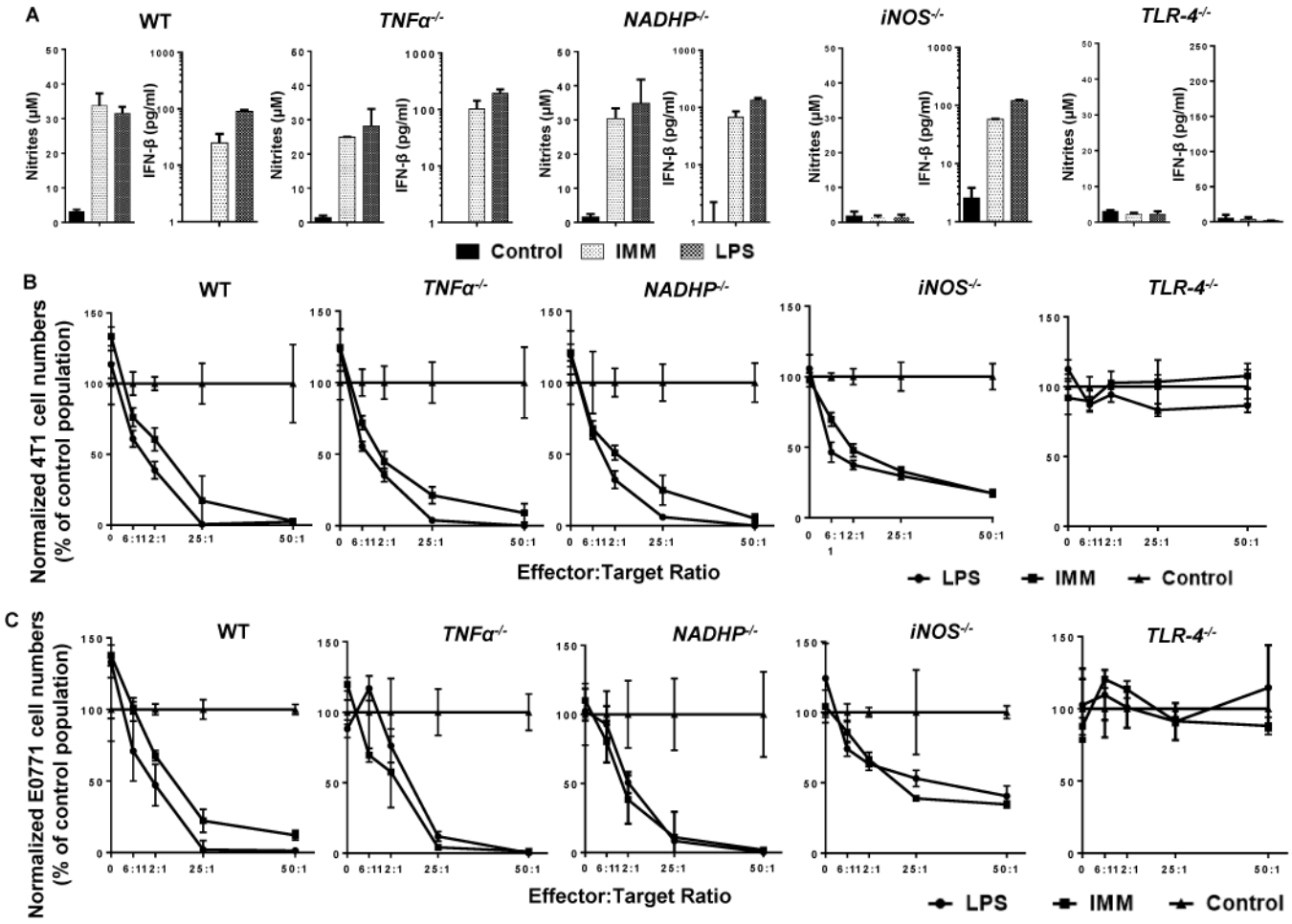

Fig. 7. knockout (KO) of *iNOS* and *TLR4* abrogated the ability of TLR4 activated BMDC to inhibit the growth of cancer cells partially and completely, respectively. (A)

Production of Nitrites and IFN-β by BMDC isolated from different KO mice, non-activated or activated with IMM (5μg/ml) or LPS (5μg/ml). Concentrations of nitrites and IFN-β in BMDC conditioned media were measured. **(B)** Growth of 4T1 breast cancer cells (H-2^d) cocultured with BMDC isolated from different mice KO for indicated gene and activated with IMM (5μg/ml) or LPS (5μg/ml). **(C)** Growth of E00771 breast cancer cells (H-2^b) cocultured with BMDC isolated from different KO mice for indicated gene activated with IMM (5μg/ml) or LPS (5μg/ml).

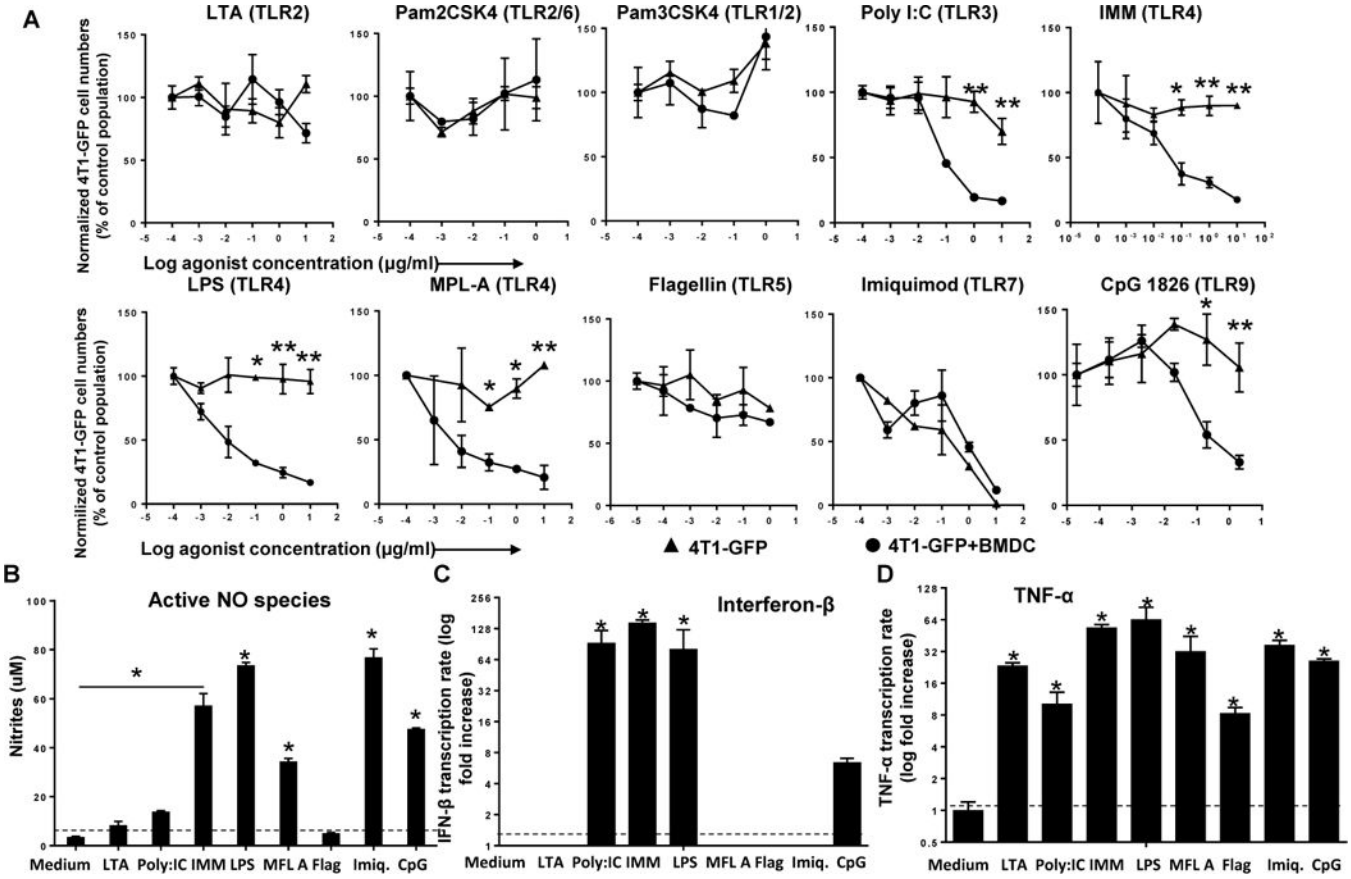

Fig. 8. TLR-agonists, inducing secretion of Peroxynitrites or Interferon-β are associated with cytotoxic properties of activated dendritic cells

(**A**) 4T1-GFP were co-cultured without or with BMDC at effector: target ratio of 1:50 for 5 days. Cell were cultured in the presence of various TLR agonists – LTA, Pam2CSK4, Pam3CSK4, Poly I:C, IMM, LPS, Monophosphoryl Lipid-A (MPL-A), Imiquimod, Flagellin, CpG 1826 at different concentration from 10pg/ml to 10 μg/ml. (**B**) Production of Nitrites by BMDC activated with different TLR agonists at concentration of 1 to 10 μg/ml (maximum concentration of agonist presented in **A**). (**C**) Up-regulation of IFN-β mRNA transcription in BMDC activated with different TLR agonists at concentration of 1 to 10 μg/ml (maximum concentration of agonist presented in **A**). (**D**) Up-regulation of TNF-α mRNA transcription in BMDC activated with different TLR agonists at concentration of 1 to 10 μg/ml (maximum concentration of agonist presented in **A**).

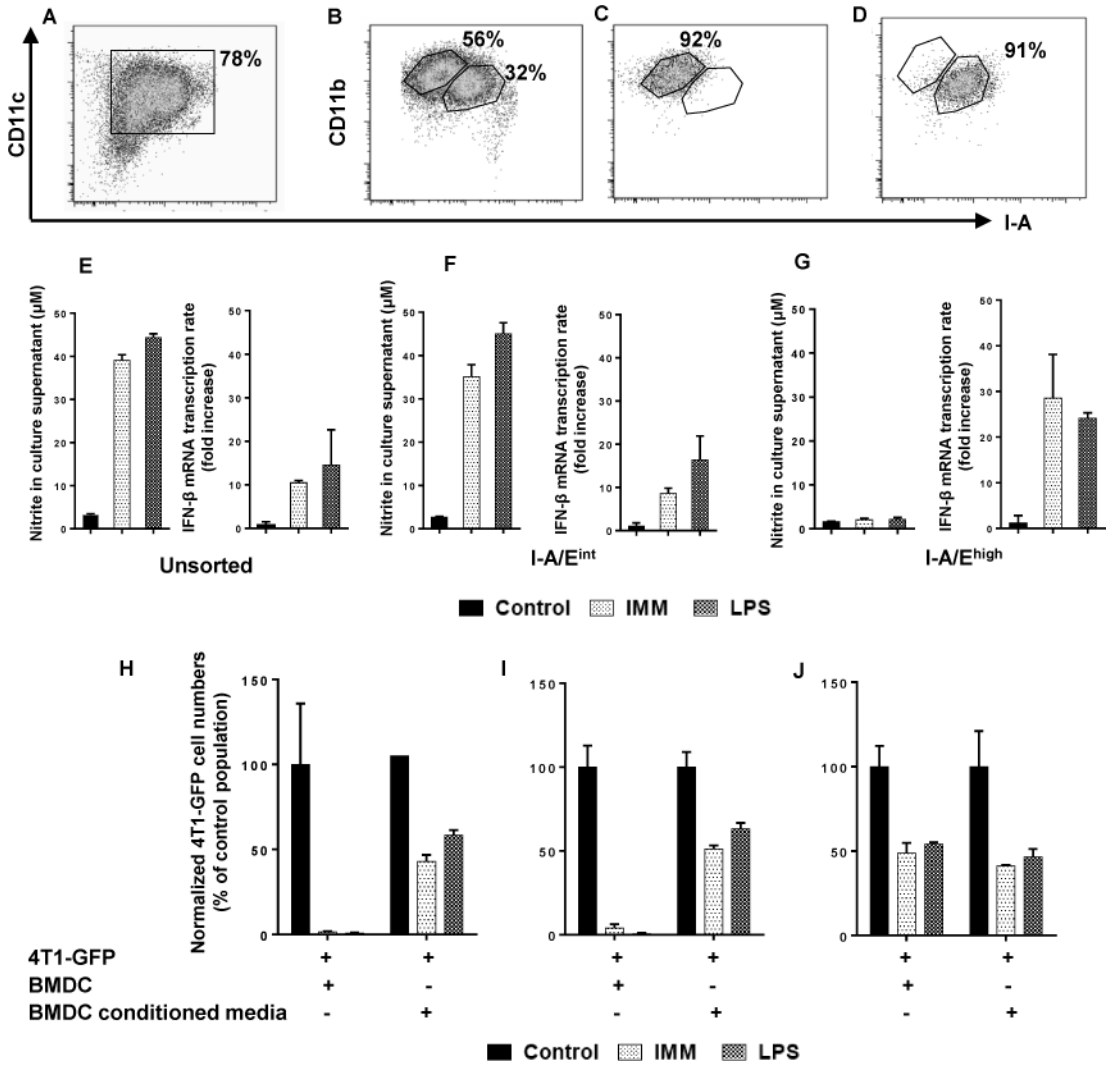

Fig. 9. Conventional BMDC comprise two subpopulations of cells with different anti-4T1 properties. (A-D)

Gating strategy of sorting of $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$ or $CD11c^{+}CD11b^{+}I\text{-}A/E^{med}$ cells **(B)** and purity of sorted cells **(C, D)**. **(E-G)** Production of nitrites and up-regulation of IFN-β transcription in conventional unsorted BMDC **(E)**, $I\text{-}A/E^{int}$ **(F)** and $I\text{-}A/E^{high}$ **(G)** subpopulations of BMDC activated with IMM or LPS. **(H-J)** Suppression of 4T1-GFP growth by conventional BMDC **(H)**, $CD11b^{+}I\text{-}A/E^{Int}$ subpopulation of BMDC **(I)** and $CD11b^{+}I\text{-}A/E^{high}$ subpopulation of BMDC **(J)** activated with IMM or LPS or non-activated control cells. 4T1-GFP cells were cocultured with BMDC or were incubated with conditioned media of BMDC**.**

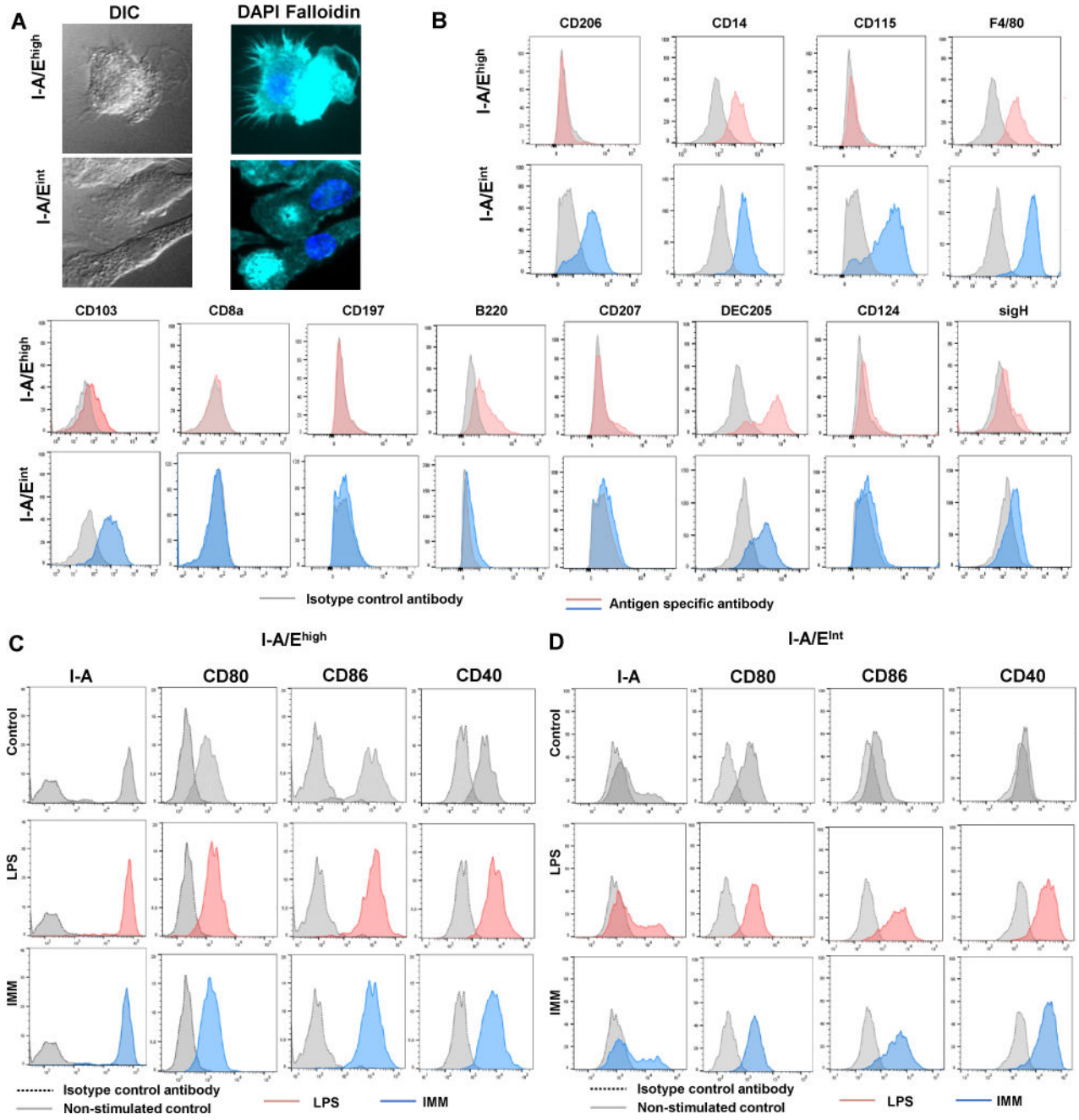

Fig. 10. Phenotypic characterization of two subpopulations of BMDC. (A) Confocal images of sorted $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$ or $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$ cells. Cells were stained with Phalloidin (Actin) and DAPI. **(B)** FACS analysis of surface proteins distribution among two subpopulations of BMDC. **(C,D)** Phenotypic changes in two subpopulations of BMDC after 24 hours of activation with IMM or LPS compared with control cells without activation. **(C)** $CD11c^{+}CD11b^{+}I\text{-}A/E^{high}$, **(D)** $CD11c^{+}CD11b^{+}I\text{-}A/E^{Int}$.