Video Article

Isolation Protocol of Mouse Monocyte-derived Dendritic Cells and Their Subsequent *In Vitro* Activation with Tumor Immune Complexes

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Abstract

Dendritic cells (DC) are heterogeneous cell populations that differ in their cell membrane markers, migration patterns and distribution, and in their antigen presentation and T cell activation capacities. Since most vaccinations of experimental tumor models require millions of DC, they are widely isolated from the bone marrow or spleen. However, these DC significantly differ from blood and tumor DC in their responses to immune complexes (IC), and presumably to other Syk-coupled lectin receptors. Importantly, given the sensitivity of DC to danger-associated molecules, the presence of endotoxins or antibodies that crosslink activation receptors in one of the isolating steps could result in the priming of DC and thus affect the parameters, or at least the dosage, required to activate them. Therefore, here we describe a detailed protocol for isolating MoDC from blood and tumors while avoiding their premature activation. In addition, a protocol is provided for MoDC activation with tumor IC, and their subsequent analyses.

Video Link

The video component of this article can be found at https://www.jove.com/video/57188/

Introduction

Since their discovery, dendritic cells (DC) have been a focus of extensive research due to their unique ability to skew T cell differentiation[1]. Over the past several decades, an extensive research effort has sought to define the various DC subsets and their function during tumor progression and immunity [2]. DCs are composed of heterogeneous cell populations that differ from each other in their pattern-recognition receptors, tissue distribution, and migratory and antigen presentation capabilities[3,4,5]. Compared to other DC subsets, monocyte-derived DC (MoDC) are far more abundant in tumors and can be easily generated from circulating or tumor-infiltrating monocytes[6,7]. Therefore, many clinical trials seeking to take advantage of their relative prevalence are based on *in vivo* and *ex vivo* manipulation of autologous MoDC in order to elicit T cell immunity [8,9].

Similarly, DC-based vaccination of experimental tumor models requires 2-3 serial injections, 5-7 days apart, of 1-2 x 10^6 activated DC pulsed with tumor antigens. Therefore, to achieve this large number of DC, most mouse studies have primarily used MoDC cultured from bone marrow (BM) precursors in GM-CSF for 7-9 days (IL-4 is not needed in the mouse setting)[10,11]. Nonetheless, given that GM-CSF knockout mice have overall normal DC compartment [12,13], and given the mixed populations obtained from that culture,[14] the physiological relevance of these DC has been called into question.

Alternatively, DC may be routinely isolated from spleen cells. However, DC comprise only about 0.3-0.8% of total spleen cells (resulting in approximately 7 x 10^5 DC/spleen), and of these cells, only $CD103^+$ DC and MoDC can migrate back to lymphoid organs. Since MoDCs comprise approximately 10-15% of splenic DC populations[15,16], most isolation protocols yield approximately 1 x 10^5 MoDC per spleen. Expansion of MoDC can be achieved by injecting transfected B16 cells that secrete GM-CSF, resulting in a 100-fold increase in splenic MoDC[17]. However, the use of MoDC for developing DC vaccines is limited since this procedure cannot be done in humans and the obtained MoDC are already highly activated.

In addition to obtaining adequate numbers of DC, another challenge for developing effective DC vaccines against autologous cancer cells involves the lack of sufficient danger signals in the tumor setting to fully activate DC. Induction of co-stimulatory signals is usually achieved through activation of pattern-recognition receptors (PRR), or c-type lectin signaling pathways[18,19,20,21]. A further approach for activating DC exploits their ability to take up antigens through interactions with surface Fcγ receptors (FcγR). Indeed, a number of important manuscripts have shown that injection of MoDC from BM precursors activated with tumor-IgG IC can prevent tumor growth in prophylactic settings, and can lead to the eradication of established tumors[22,23].

In two recent papers, *Carmi et al*. discovered that in contrast to BMDC and spleen DC, MoDC from the blood and tumors cannot respond to IgG IC without additional stimuli. This was found to be due to the presence of high intracellular levels of tyrosine phosphatases regulating FcγR signaling[24,25]. By defining a critical checkpoint in DC, this work provided an important insight into the requirements for successful DC-based vaccination. The requirement for additional stimuli to enable FcγR signaling, and presumably signaling from other lectin receptors utilizing a similar phosphorylation cascade, thus underscores the need for avoiding the priming of DC during their isolation.

Therefore, the present protocol describes the isolation of MoDC from blood and tumors, which differ markedly from BM and spleen DC, and highlights precautions worth considering during the process.

Protocol

The protocols below refer to the isolation of mouse MoDC, yet the overall principles may apply to other DC subsets cells, as well. 12 - 16-week-old C57Bl/6j mice were maintained in an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility. All protocols were approved by Stanford University and Tel-Aviv University Institutional Animal Care and Use Committee.

1. Isolation of Tumor Associated Monocyte-Derived DC

1. In a laminar flow hood, remove tumors from CO_2 euthanized mice and place tumors in RPMI medium without fetal bovine serum (FBS).
   NOTE: It is highly recommended to shave the mice and spray them with 70% ethanol before removing the tumors, to minimize potential contaminations from the fur. Be certain that the tumor does not exceed 25 - 40 mm^2 since larger tumors have fewer DC that tend to be fragile and prone to undergo cell death. If larger numbers of DC are needed, each mouse can be injected with tumors cells at multiple sites.
2. Under a sterile laminar hood, chop the tumors using surgery scissors into small pieces (approximately 1 x 1 mm^2).
   1. Add all tumor fragments from one mouse into a 30-mL sterile flat-bottom tube with magnetic stir bars, which contains 5 mL of Hank's balanced salt solution (HBSS), 2 mg/mL collagenase IV, and 0.01 mg/mL DNase I.
      Caution: Myeloid cells produce energy through glycosylation, even under steady state. Therefore, only use media that contains glucose (e.g. HBSS, RPMI, and DMEM), as glucose starvation for as little as 10 - 15 min will result in cell death and apoptosis within 24 h. Use only a low endotoxin collagenase IV, as collagenase is produced by Gram-positive bacteria (*Clostridium histolyticum)*.
3. Stir at about 200 - 400 rpm in a 37 ^oC incubator with a magnetic stirrer for 20-30 min.
4. Add 5 mL of complete media and re-suspend vigorously.
5. Filter cells through a 70-µm cell strainer. Centrifuge at 4 ^oC 400 rcf for 5 - 10 min to pellet the cells.
   Caution: Do not attempt to isolate the cells directly following enzymatic digestion, as some tumors are necrotic and contain relatively large amounts of cell debris or extracellular matrix. Apply cells on 15% density gradient medium to obtain only the cells.
6. Suspend 9 mL of density gradient medium with 1 mL of 10x PBS to adjust osmolality, and to obtain 100% Percoll stock solution. Mix 1.5 mL density gradient medium stock solution with 8.5 mL HBSS and mix it vigorously with tumor-derived cell pellet. Gently layer 2 mL of DMEM on the top of 15% density gradient medium and centrifuge at 400 rcf for 20 min at room temperature. Discard the supernatants, as the cells will form a pellet at the bottom of the tube.
   1. Wash the pelleted cells twice by re-suspending them in 10 mL HBSS containing 2% FBS, 1% penicillin/streptomycin, and 10 mM EDTA (Isolation Buffer), and centrifuge at 4 ^oC 400 rcf for 5-10 min to pellet the cells.
   2. Count cells on a hemocytometer using trypan blue under a light microscope.
7. Re-suspend 1 x 10^8 cells in 1 mL isolation buffer and incubate them at 4 ^oC with 30 µL of $CD11b^+$ conjugated magnetic beads for 15 min.
   Caution: Avoid the use of CD11c-conjugated beads, as this will activate the DC and induce cell death. Do not attempt to block FcγRII and FcγRIII with anti-CD16/32 antibodies. Blocking antibodies ligate FcγR and induce strong phosphorylation of MAP kinases and prime the DC.
   1. Remove the excess unbound beads by adding 9 mL of isolation buffer and centrifuge cells at 400 rcf for 5 - 10 min at 4^oC.
8. Aspirate the supernatant and re-suspend cells in 1 mL isolation buffer. Apply the cells on a prewashed magnetic column. Wash the column twice with 3 mL of isolation buffer.
   1. Remove the column from the magnet. Pipette 6 mL of isolation buffer onto the column and flush out the magnetically-labeled cells into a sterile collection tube by pushing the plunger. Centrifuge cells at 400 rcf for 5 - 10 min at 4 ^oC.
   2. Re-suspend cells at 100 µL per 1 x 10^7 cells and stain with the following fluorophore-conjugated antibodies: Linage negative (TCRb, Siglec F, B220, CD19, FceRI and Ly6G), MHCII, Ly-6C.
9. Sort cells by gating the small cells, using side scatter (SSC) and forward scatter (FSC) and culture in complete medium supplemented with 5 ng/mL GM-CSF. Incubate cells for 1 hour at 3 7 ^oC in order to allow macrophages to adhere the plate. Then, transfer the loosely and non-adherent cells to a new culture dish.
   NOTE: Mouse MoDC are defined as $CD11b^+/CD11c^+/MHCII^{hi}/Ly6C^{lo/int}$ [7,26,27]. Expression of Ly6C may vary dramatically between tumor models, and in some models (e.g. LMP) MoDCs completely lack Ly6C expression. Overall, one 100 mm^3 B16F10 tumor typically contains 5 x 10^4 of DC, resulting in approximately 4 - 5 x 10^6 total cells. Of these cells, 10 - 15% are immune cells and 8 - 10% are MoDC. Increasing the DC numbers can be achieved by injecting mice with tumors at multiple sites. Sort only small SSC/FCS cells, as other myeloid cells can express markers such as CD11c and Ly6C.
   Optional: Sort the tumor-infiltrating monocyte as small SSC/FSC cells that express $Ly6C^{hi}$ and are negative for MHCII. Afterward, culture monocytes *in vitro* with 50 ng/mL GM-CSF to obtain DC.

2. Isolation of Monocyte-derived DC from Peripheral Blood

NOTE: Since mature MoDCs are relatively rare in mouse blood, the protocol below refers to their derivation *in vitro* from sorted monocytes.

1. To increase the levels of monocytes in blood, anesthetize mice with 4% isoflurane and inject them subcutaneously (s.c.) with 1 µg of GM-CSF in 50 µL PBS.
2. After 1-2 h, sacrifice mice using CO_2.
   Caution: Do not sacrifice mice by cervical dislocation, as this will cause internal bleeding.
3. Immediately after euthanization,spray the mouse with 70% ethanol and remove the skin that covers the heart using surgical scissors, under a sterile laminar flow hood. Clean the scissors with ethanol and cut the right atrium of the heart.Slowly, flush the heart through the right ventricle with 20 mM EDTA HBSS using a 10-mL syringe and 25-G needle.
   1. Collect the blood with a sterile syringe from the pleural cavity into a sterile tube containing heparan sulfate and EDTA. Continue to flush the heart until the liver and lungs become pink or white.

4. Apply blood onto a density gradient mediumand centrifuge at 400 rcf at room temperature for 15 min with low brake.
   Caution: Use endotoxin-free density gradient medium (usually less than 0.12 EU per mL).
   1. Collect mononuclear cells into a new tube. Wash the cells by re-suspending in 10 mL HBSS containing 2% FBS, 1% penicillin/streptomycin and 10 mM EDTA (isolation buffer), and then centrifuging at 4 ^{o}C 400 rcf for 5-10 min to pellet the cells.
      Caution: Use only media that contains at least 1 g/L of glucose.

5. For CD11b positive selection, re-suspend 1 x 10^8 cells in 1 mL isolation buffer and incubate them for 15 min with 50 µL of CD11b-conjugated magnetic beads at 4 ^{o}C.
   1. Wash excess beads by adding 9 mL of isolation buffer and centrifuging at 400 rcf for 5-10 min at 4 ^{o}C. Aspirate the supernatants and re-suspend the cells in 1 mL isolation buffer. Apply the cells on a prewashed magnetic column according to manufacturer's instructions.
   2. Remove the column from the magnet, pipette 6 mL of isolation buffer onto the column, and flush out the magnetically-labeled cells into a sterile collection tube by pushing the plunger. Centrifuge cells at 400 rcf for 5-10 min at 4 ^{o}C. Wash the column twice with 3 mL of isolation buffer.
   3. Re-suspend cells at 1 x 10^7 cells/100 µL isolation buffer and stain with the following fluorophore-conjugated antibodies: 0.1 µg CD115, 0.25 µg MHCII, and 0.1 µg Ly-6C/1 x 10^6 cells.

6. Sort cells by gating on small side scatter (SSC) and small forward scatter (FSC) cells.
   NOTE: Mouse inflammatory monocytes are generally defined as $CD115^+/MHCII^{lo/neg}/Ly6C^{hi}$, and patrolling monocytes are defined as $CD115^+/MHCII^{lo/neg}/Ly6C^{neg}$ [4,5]. In cases where no separation between the two subsets is needed, total $SSC^{lo}/FCS^{lo}/CD115^+/MHCII^{lo}$ cells can be sorted. Both naïve and tumor-bearing mice contain approximately 5 - 8 x 10^4 MoDC per 1 mL of blood and up to 4 - 5 x 10^5 MoDC following injection of GM-CSF. Of them, approximately 70% are inflammatory monocytes and 30% are patrolling monocytes.
7. Plate the cells at a concentration of 1 x 10^6 cells/mL in a complete media supplemented with 20 ng/mL GM-CSF. After 1 day, transfer the non-adherent and loosely adherent cells to a new plate and culture for an additional 4-5 days.
   Caution: DC media should not be supplemented with pyruvate.

3. Preparation of Tumor-IgG Immune Complexes

1. Culture tumor cells in 75 cm^2 culture flask to 70% confluence in complete DMEM media.
   1. Add 2 mL of 0.25% trypsin/EDTA to detach cells from the culture flask and monitor cell morphology under a light microscope to avoid over-trypsinization.
   2. Add 8 mL of complete culture media (per 2 mL of trypsin) to inhibit trypsin digestion, and centrifuge at 4 ^{o}C, 400 rcf for 5-10 min to pellet the cells.
      NOTE: Make sure to check tumor cells for *Mycoplasma* using a commercial PCR kit. Test for the presence of Gram-negative bacteria and fungal endotoxins using *Limulus Amebocyte Lysate* (LAL) assay[28]). Serum must be filtered through 0.22 µM and tested for the 9 Code of Federal Regulations viruses. Additionally, test the culture media and serums by dropping 100 - 200 µL onto LB agar or broth, and culture for 2 days at 37 ^{o}C.
   3. Wash cells again from trypsin and serum remains by re-suspending them in 10 mL PBS and centrifuge at 400 rcf for 5 - 10 min. Aspirate supernatant and repeat the wash 2 more times.

2. Fix cells in 1.8% buffered paraformaldehyde for 10 min at room temperature.
   1. Wash the cells by re-suspending them in 10 mL PBS and centrifuge at 4 ^{o}C 400 rcf for 5 - 10 min.
   2. Aspirate supernatants and repeat wash two more times.
      Optional: For tumor uptake assays, cells can be labeled by incubating them for 5 min at 37 ^{o}C in PBS containing 1 µM carboxyfluorescein succinimidyl ester (CFSE). CFSE is then quenched with complete media for 10 min in ice. The cells should be washed extensively in PBS containing 2% serum to remove residual dye.

3. Re-suspend cells in FACS buffer (PBS supplemented with 2% FCS + 5 mM EDTA) and 0.5 µg/mL of anti-CD16/32 in order to block potential non-specific protein-protein interactions. Plate in a U-shape 96 wells/plate at a concentration of 1 x 10^5 cells per 100 µL.
   1. Add different dilutions of tumor-binding antibodies ranging from 5 µg - 5 ng/1 x 10^5 cells. Incubate the plate on ice for 15-20 min.
      NOTE: IgG antibodies were isolated from the serum of naïve 20-24 weeks old female mice on protein A columns, as described[24].

4. Wash cells by adding 150 µL of PBS and centrifuging the plate at 4 ^{o}C 400 rcf for 5 - 10 min.
   1. Discard the supernatants and repeat the wash twice. Re-suspend cells in 100 µL FACS buffer containing fluorophore-conjugated secondary antibody. Incubate plate on ice for 20 min.
   2. Wash cells by adding 200 µL of FACS buffer and centrifuging the plate at 400 rcf for 5 - 10 min. Discard the supernatants and repeat wash.
   3. Analyze tumor binding by flow cytometry and determine the minimal concentration required to coat the cells.

NOTE: Antibodies that do not increase mean fluorescence intensity (MFI) of stained tumor cells by at least fivefold over isotype control should not be used in subsequent functional assays.

4. Activation of MoDC with Tumor-IgG IC

1. Coat tumor cells with minimal IgG concentration, as described in sections 3.1 through 3.4.3
    1. One day before activating MoDC with tumor IC, replace MoDC culture media, which contains GM-CSF. To do so, gently aspirate the media and wash the cells once with pre-warmed complete culture media.
    NOTE: Isolated mature tumor-associated MoDC should be cultured for at least 2 - 3 h (or even overnight) after sorting in complete media without GM-CSF, and before activating them with IC.
    2. For tumor uptake analyses, add the CFSE-labeled tumor IC to MoDC at a ratio of 1:5 (IC:MoDC) and incubate overnight for 12 - 16 h in 1 mL of complete media per 1 x 10^6 DC.
    3. For FACS analyses of MoDC activation experiments, add tumor-IC at 1:1 (IC:MoDC) ratio and incubate overnight for 12 - 16 h.
    NOTE: It is highly recommended to include a positive control well, in which MoDC are stimulated with 1 µg/mL of LPS or other TLR agonists.
    4. Following overnight activation, aspirate the supernatants and wash cells gently three times with isolation buffer, or 10 mM EDTA HBSS.
    5. To detach DC from the plate, incubate cells for 2 - 3 min in 1 mL HBSS containing 10 mM EDTA, and detach cells by vigorous pipetting.
    6. Centrifuge cells at 400 rcf and re-suspend 1 x 10^6 DC in 90 µL PBS supplemented with 2% FCS, 5 mM EDTA (FACS buffer) and 0.5 µg of blocking antibodies. Incubate on ice for 5 - 10 min.
    7. Add 10 µL of staining antibodies mixture to the cells and incubate on ice for 15 min.
    8. Add 2 mL FACS buffer to cells and centrifuge at 4 ^oC 400 rcf for 5 - 10 min.

2. Re-suspend cells in 200 µL FACS buffer.
    1. Add 0.5 - 1 µg/mL of DAPI 1 - 2 min before running the samples, to exclude dead cells from analyses. Do not over-incubate DAPI, as MoDC will take it up within 10 - 15 min.

Representative Results

We initially compared the capacity of antibodies from naïve syngeneic and allogeneic mice to bind to tumor cells. To this end, B16F10 and LMP tumor cell lines were fixed in paraformaldehyde and washed extensively. B16F10 is a melanoma cell line, which was originally isolated from lung metastases in C57Bl/6 mice. LMP is a pancreatic tumor cell that was isolated from KrasG12D/+, LSL-Trp53R172H/+, and Pdx-1-Cre mice, and grows steadily in 129F1 mice. To obtain IC, tumor cells were incubated for 20 min on ice with 2 µg of syngeneic or allogeneic IgG per 1 x 10^5 tumor cells. IgG and IgM antibodies were isolated from the circulation of naïve 20-24-week-old mice on protein A, followed by size-exclusion chromatography as described[24]. Cells were then washed and stained with PE-conjugated rat anti-mouse IgG secondary antibody, and the mean fluorescent intensity was analyzed in a flow cytometer. As indicated in **Figure 1A**, IgG antibodies from C57Bl/6 allogeneic mice bound LMP tumor cells *ex vivo* far more effectively than antibodies from 129S1 syngeneic mice. Similarly, staining of fixed B16F10 with 129S1 allogeneic IgG was more than tenfold higher, compared to staining with syngeneic IgG from naïve C57Bl/6 mice. Interestingly, mice bearing B16 tumors failed to produce antibodies with the similar binding capacity to that of allogeneic mice, even during the tumor progression (**Figure 1B**).

We next sought to compare the IC response of MoDC from blood and tumors to that of DC from the spleen and BM. To isolate tumor-associated MoDC, B16F10 tumors were enzymatically dissociated to obtain single cell suspensions. Immune cells were enriched using CD45-magnetic beads, and MoDC were further sorted as $SSC^{lo}/FSC^{lo}/CD11c^{+}/MHCII^{+}/Ly6C^{lo}$ by FACS (**Figure 2A**). It is noteworthy that different tumor DC may have different markers that define them. To isolate MoDC from the blood, circulating monocytes were enriched by CD11b-conjugated magnetic beads and further sorted as $SSC^{lo}/FSC^{lo}/ CD115^{+}/MHCII^{neg/lo}$. Cells were then cultured for 1 day in GM-CSF, and the non-adherent and loosely adherent cells were transferred into a new plate and cultured for an additional 4-5 days to obtain MoDC (**Figure 2B**). As a reference point we used BMDC, serving as the "gold standard" DC for many functional assays, as well as splenic MoDC, which reflect a more physiological DC subset. BMDC were obtained by sorting the BM pro-monocytes ($CD11b^{+}/Ly6C^{hi}/CD115^{hi}/MHCII^{neg}$), followed by their culturing for 7 days with GM-CSF, as described[24]. Splenic MoDC were isolated from the single cell suspension, obtained by mashing the spleen through a cell strainer (pre-incubation with collagenase is not required for splenic MoDC), followed by enrichment with CD11b-conjugated magnetic beads. Cells were then sorted as $SSC^{lo}/B220^{neg}/NKp46^{neg}/CD3^{neg}/Gr1^{neg}/F4/80^{neg}/MHCII^{hi}/CD11c^{hi}$ and cultured for 1 hour in complete RPMI at 37^oC to restore baseline activity.

To investigate the effect of IgG IC on MoDC activity, we incubated the isolated DC subsets overnight with fixed tumor cells or with fixed tumor cells pre-coated with allogeneic IgG. Activation of BMDC or splenic DC with IC resulted in increased CD86 and MHCII expression, unlike MoDC, or tumor-associated MoDC (**Figure 3A**). The ability to uptake CFSE-stained tumor-derived proteins, with or without allogeneic IgG, was also compared. As observed by confocal microscopy, splenic DC showed superior ability to internalize the tumor-derived proteins (**Figure 3B**).

Taken together, these results suggest that the tumor DC and MoDC respond differently than splenic DC and BMDC to activation with alloIgG-IC. Therefore, vaccination strategies that wish to activate tumor DC cannot be based solely on the activation patterns of BMDC.

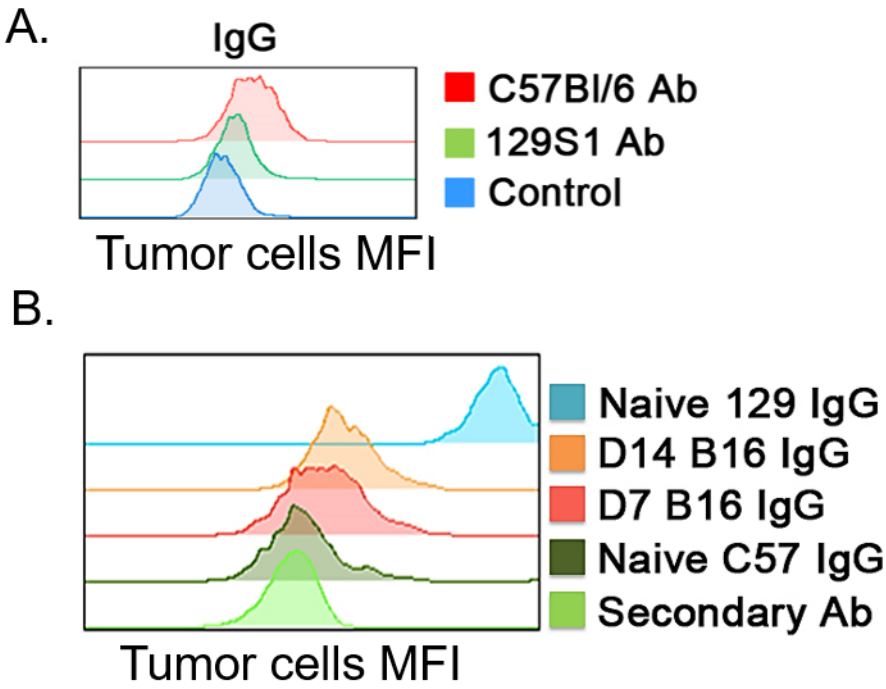

Figure 1: Allogeneic mice have naturally-occurring tumor-binding IgG antibodies in their circulation: A. Mean fluorescence intensity (MFI) of LMP tumor cells stained with IgG antibodies isolated from the blood of syngeneic (129S1) and allogeneic (C57Bl/6) naïve mice. **B.** Mean fluorescence intensity (MFI) of B16F10 tumor cells incubated with IgG antibodies isolated from the circulation of syngeneic tumor-bearing mice (C57Bl/6), or allogeneic (129S1) naïve mice. This figure has been modified from extended data 2A and 2B Carmi Y *et al*. *Nature* 521 7550:99-104, 2015[24].

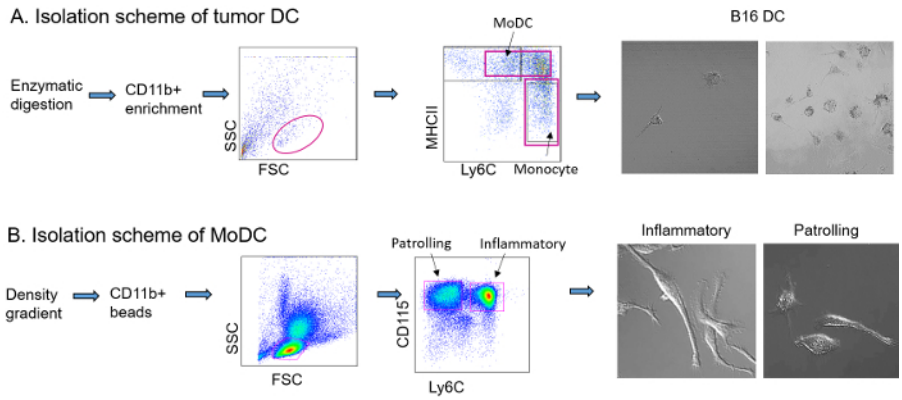

Figure 2: Sorting scheme of mouse MoDC from blood and B16 tumors. A. Isolation and sorting scheme of MoDC cultured from mouse monocytes. Confocal microscopy DIC image of inflammatory and patrolling monocytes after 4 days in culture (x400). **B.** Isolation and sorting scheme of tumor-associated MoDC from B16 tumors. Representative confocal microscopy DIC image of MoDC isolated from B16 tumors after overnight culture (x400). This figure has been modified from Supplementary figure 1 Carmi Y *et al. JCI insight* 1:18:e89020, 2016[25]. Please click here to view a larger version of this figure.

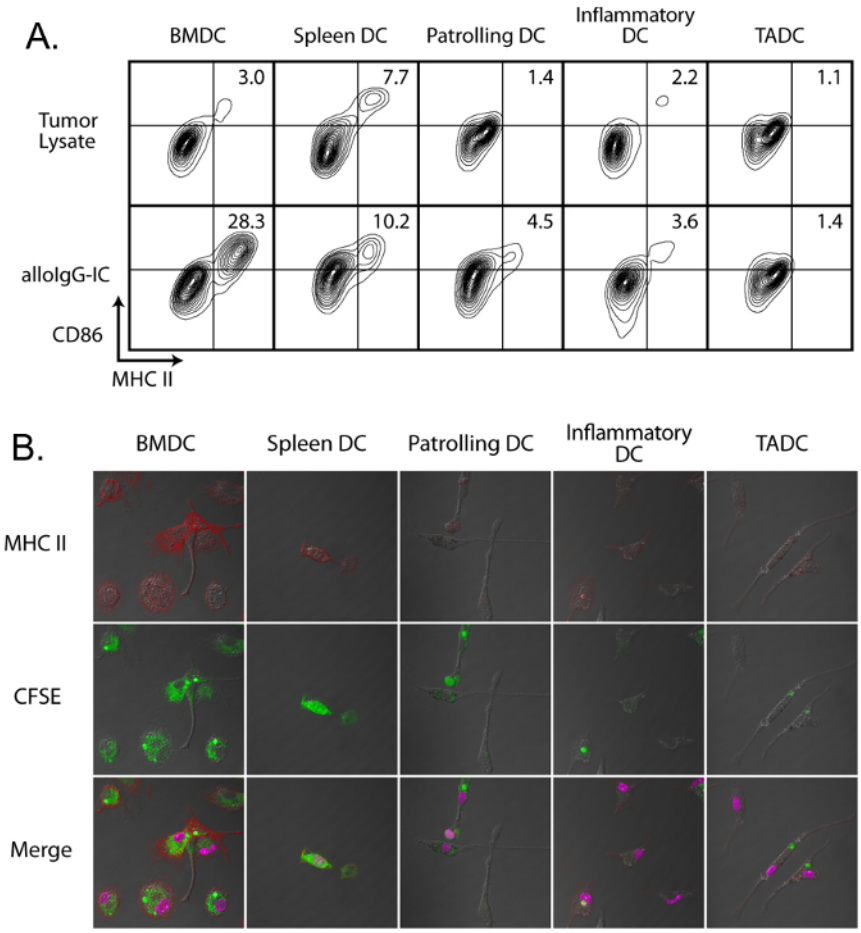

Figure 3: MoDC from the spleen and BM display different patterns of activation than tumor and blood MoDC following incubation with IC. A. Flow cytometric analysis of MHCII and CD86 expression by DC incubated overnight with tumor-IgG IC. **B.** Confocal immunohistochemistry of tumor uptake (green) and MHCII expression (red) by DC incubated overnight with CFSE-labeled tumor IC. This figure has been modified from figures 3A and 3DCarmi Y *et al.* Akt and SHP-1 are DC-intrinsic checkpoints for tumor immunity. *JCI insight* 1:18:e89020, 2016[25]. Please click here to view a larger version of this figure.

Discussion

Given the large number of DC required for vaccinating mice (approximately 2-4 x 10^6 DC per one mouse), most of the vaccination strategies in mice are based on isolation of DC from BM and spleen followed by their *ex vivo* activation. However, attempts to activate tumor DC *in vivo*, using the same conditions for activating spleen and BM DC, have often been unsuccessful in producing effective immunity. In two subsequent publications, Carmi *et al*. have found that blood and tumor MoDC differ significantly from spleen and BM DC, given that they bear naturally high intrinsic levels of tyrosine phosphatase and require priming prior to activation with IC[24,25]. Moreover, these results further stress the need for taking extra precautions in order to maintain DC in an activation state that better reflects their physiological status. Therefore, the present protocol seeks to provide a detailed description of the isolation process of MoDC from tumors and the circulation, and to highlight the steps that may result in their premature activation.

First, in order to obtain sufficient numbers of DC from blood and tumors, there is a need for a much greater number of mice compared to that of protocols for isolating BMDC. To increase the yield of the DC we use 16-week-old mice, which have larger blood volumes and overall cell counts. We routinely get 5-8 x 10^4 MoDC per 1 mL of blood without injection of GM-CSF, and 4-5 x 10^5 MoDC following injection. For tumor DC, we obtain approximately 6-8 x 10^4 MoDC from a 100 mm^3 tumor, though the number may vary between individual mice and between tumor models. Increasing tumor size results in a lower DC yield, as a 1,000 mm^3 tumor will have only about 5,000 DC. Instead, we inject mice with tumor cells at multiple sites (typically 4-6), thereby obtaining up to 4 x 10^5 MoDC per mouse.

Furthermore, we recommend that prior to their experimental use, antibodies, cell lines, tubes and general lab reagents should be tested for endotoxins by LAL assay, and by plating the media on AC bacterial agar. Tumor cell lines are often infected with *Mycoplasma* and should therefore be tested by PCR prior to their incubation with DC. Use of crude collagenase preparations, such as Types 1 and 4, is a primary source of endotoxins due to isolation of the collagenases from *Clostridium histolyticum*. Standard preparations of collagenase may contain as much as 10 EU/mg of endotoxins, which is about 1-2 ng of endotoxin per mL of digestion mixture. Jahr *et al.* have found that collagenase preparations containing 2.7-6.7 ng/mL of endotoxins will induce 1,415-3,967 ng/mL of IL-1β following culture with PBMC[29]. Indeed, priming of DC is routinely done with 1 ng of LPS per mL media, yet as little as 100 picograms/mL is sufficient to prime them[30,31,32]. Another potential source of endotoxins

is FBS, which may contain 25 EU/mL or more of endotoxins, or less than 10 EU/mL. Assuming that the culture media contains 10% FBS, the endotoxin load could reach 0.5 ng/mL, which is sufficient to prime DC.

In addition, many protocols use anti-CD16/32 antibodies to block Fc receptors as a means of increasing the specificity of their antibody panel prior to sorting. Nonetheless, cross-linking of FcγRIII (CD16) leads to phosphorylation of Syk/ZAP-70 and PLC-γ, release of intracellular Ca^{2+} [33],and phosphorylation of P38 and ERK1/2in both human[34] and mouse monocytes[35]. In our hands, addition of anti-CD16/32 to MoDC cultures consistently induces a strong phosphorylation of P38 and ERK1/2 kinases in DC within one minute of exposure. We, therefore, strongly suggest avoiding the use of anti-CD16/32 when isolating MoDC for *in vitro* functional assays.

Another common protocol uses enrichment of DC by CD11c magnetic beads prior to their sorting by FACS. CD11c is a type I transmembrane glycoprotein that recognizes a variety of ligands, including fibrinogen, LPS, type I collagen,and the inactivated C3b subunit. Rezzonico *et al.* have shown that ligation of CD11c with antibodies induces potent NFκB activation and secretion of chemokines[36]. In our experience, immobilized anti-CD11c antibodies induce cell activation and apoptosis, while the use of their soluble form in FACS sorting does not induce phosphorylation of either P38 or ERK1/2.

Overall, this protocol is designed to achieve isolation of MoDC with as little activation as possible, so that their subsequent activation *in vitro* reflects the conditions required for their activation *in vivo*.

Disclosures

All authors declare they have no conflict of interest and that they have nothing to disclose.

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None

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