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Role of heterogeneous cell population on modulation of dendritic cell phenotype and activation of CD8 T cells for use in cell-based immunotherapies

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Abstract

Dendritic cell (DC)-based immunotherapies have much utility in their ability to prime antigen-specific adaptive immune responses. However, there does not yet exist a consensus standard to how DCs should be primed. In this study, we aimed to determine the role of heterogeneous co-cultures, composed of both CD11c⁺ (DCs) and CD11c[−] cells, in combination with monophosphoryl lipid A (MPLA) stimulation on DC phenotype and function. Upon DC priming in different co-culture ratios, we observed reduced expression of MHCII and CD86 and increased antigen uptake among CD11c⁺ cells in a CD11c[−] dependent manner. DCs from all culture conditions were induced to mature by MPLA treatment, as determined by secretion of pro-inflammatory cytokines IL-12 and TNF- α . Antigen-specific stimulation of CD4⁺ T cells was not modulated by co-culture composition, in terms of proliferation nor levels of IFN- γ . However, the presence of CD11c[−] cells enhanced cross-presentation to CD8⁺ T cells compared to purified CD11c⁺ cells, resulting in increased cell proliferation along with higher IFN- γ production. These findings demonstrate the impact of cell populations present during DC priming, and point to the use of heterogeneous cultures of DCs and innate immune cells to enhance cell-mediated immunity.

Graphical abstract

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Conflict of Interest

The authors declare no conflict of interest.

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Keywords

Dendritic cells; Toll-like Receptors (TLRs); Immunotherapy; Cell activation; T cells; Priming protocol; Bone marrow

1. Introduction

Dendritic cells (DCs) are unique immune cells that bridge the innate and adaptive immune systems through their ability to effectively present antigen to T cells.[1] DC-based immunotherapies prime DCs *ex vivo* with a specific antigen, induce their maturation, and then re-administer them back into the body where they will home to the lymph nodes and activate the adaptive immune system.[2] Many DC-based therapies have moved into clinical trials and demonstrated safety and the ability to elicit antigen-specific immune responses. However, these studies have shown only modest performance in objective clinical outcomes such as tumor response rates.[3,4] While the exact mechanism for these observed outcomes has not been fully elucidated, using DC-vaccines in combination with other immunoregulatory therapies as well as generating DCs that are more immunogenic and functional for administration are active areas of investigation.[5] The efficacy of DC-vaccines to generate desired immune responses has been probed with respect to parameters such as type of antigen loaded, maturation stimuli used, route of administration, and the subsets of DCs utilized.[4,6,7] Overall, generation of these DC-therapies has not been optimized and its achievement is critical to understand how to generate more effective immunotherapies.

One strategy to improve DC-therapies involves the use adjuvants, such as toll-like receptor (TLR) agonists, to activate DCs and induce their maturation and migration to lymph nodes where they can interact with T cells.[8–10] This stimulation renders DCs with the ability to effectively activate encountered T cells and induce lineage commitment and effector responses. Specifically, the TLR4-agonist monophosphoryl lipid A (MPLA) has been shown to increase the function of antigen presenting cells (DCs and macrophages) to induce T cell proliferation and polarization into T-helper 1 cells (T_H1), which are important in fighting intracellular pathogens and tumors.[11,12] Many DC-therapy clinical trials have shown a superiority of mature over immature DCs in terms of clinical outcomes.[13,14] Thus, in adoptive cell transfers, DCs are commonly pulsed with stimulating factors, such as TLR ligands, to induce their maturation and enhance their effectiveness.[15]

In the generation of these therapies for pre-clinical studies, DCs are frequently derived from monocyte precursors in the bone marrow using granulocyte macrophage-colony stimulating factor (GM-CSF), which is a growth factor that generates a heterogeneous population of both CD11c+ (DCs) and CD11c- cells (non-DC monocytes and granulocytes).[16–18] For application in human clinical trials, DCs are commonly derived from blood monocytes.[19] Crosstalk between cell populations, particularly between immune cell populations, has been shown to exist dynamically. Such communication can control immune responses and influence the balance between inflammation and tolerance.[20–23] For instance, paracrine signaling of cytokines and other soluble factors can activate nearby cells and propagate the immune response.[24,25] Additionally, direct cell-cell contact via receptor binding can also induce intracellular signaling that leads to activation and suppression of cellular programs or the production of immunological factors in a variety of immune settings.[25–27] The effect of such cell crosstalk can be observed in the context of DC-based therapies, in which the use of the bone marrow-derived heterogeneous CD11c+ and CD11c- population significantly increased the percentage of tumor-free mice after melanoma challenge compared to the purified DC population. This study demonstrated the importance of the presence of CD11c- cells in achieving the desired immune response of restricting tumor growth.[17]

Extensive crosstalk and cell-cell communication could occur in this heterogeneous environment to modulate DC maturation, antigen uptake, processing, and presentation, which are critical for the success of DC-based therapies. Although this parameter has shown to be necessary to induce antitumor protection in mice *in vivo*, the role of cell populations present during *ex-vivo* priming is still significantly understudied. Moreover, how DC phenotype and immunogenicity is effected by TLR activation when DCs are cultured in a heterogeneous cell population has not been investigated.

We aim to further exploit the use of heterogeneous co-cultures to strengthen DC-therapies. Here, we explore how different percentages of CD11c- cells present during DC priming and stimulation influence DC phenotype and capability of activating adaptive immune responses. Such findings can impact priming protocols for cell-based therapies to produce superior DCs, thereby potentially leading to improved clinical outcomes. In this study, we hypothesized that DC phenotype, antigen uptake, and functional ability to stimulate T cells are controlled by MPLA stimulation in combination with the composition of the CD11c+/- population in culture. Interestingly, the heterogeneous population of MPLA-treated CD11c+/- cells at specific ratios negatively affected DC maturation and preferentially induced CD8+ T cell proliferation via cross-priming. Hence, we demonstrate that an *ex vivo* DC preparation protocol based on a combination of heterogeneous cells and TLR stimulation can possibly be a potent strategy to enhance cellular immunity against tumors or infections.

2. Materials and Methods

2.1 Derivation of bone marrow DCs and CD11c+ isolation

Bone marrow-DCs were derived as previously described.[29] Briefly, tibias and femurs were collected from the hind limbs of 8–12 week old female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and the bone marrow was flushed out. Red blood cells were lysed and the remaining cells were cultured on petri dishes in complete cell culture media: RPMI-1640

(Cellgro, Manassas, VA), 2 mM L-glutamine (Cellgro), 10 mM HEPES, 1x non-essential amino acids, 1 mM Na Pyruvate, 55 μ M 2-mercapthoethanol, 1% penicillin/streptomycin, and 10% heat-inactivated fetal bovine serum (all from Invitrogen, Carlsbad, CA) with 20 ng/mL GM-CSF (Peprotech, Rocky Hill, NJ). After 3, 5, and 7 days of culture the cells were supplemented with new media and GM-CSF (at 20 ng/mL). On day 8, all cells were detached, collected, and magnetically labeled with anti-CD11c microbeads (Miltenyi Biotec, Auburn CA). Labeled cells were flowed through a magnetic column and CD11c⁺ cells were isolated by positive selection. The retained fraction (CD11c enriched) was separated from the eluted fraction (CD11c reduced) and used for further experimentation. Immediately after separation, the CD11c enriched isolate was found to be 70.98% CD11c⁺ $\pm$ 20.22 and the CD11c reduced isolate was found to be 26.36% CD11c⁺ $\pm$ 7.90 as identified by flow cytometry (FACSCanto II). Data were analyzed using FlowJo software (Tree Star).

2.2 CD11c⁺ DC phenotype analysis with and without TLR stimulus

After CD11c⁺ cell isolation, cells were seeded in 24-well plates at varying ratios of cells from the CD11c enriched isolate to cells from the CD11c reduced isolate. Ratios of 8:1, 1:1, and 1:8 were investigated. Wells with only CD11c enriched and reduced isolate cells were included as controls. Total cell number was kept constant for all ratios at 250,000 cells/well. After seeding, cells received either media only or stimulation with MPLA (10 μ g/mL) (InvivoGen, San Diego, CA). Lipopolysaccharide (LPS) stimulation (1 μ g/mL) of CD11c enriched cells was included in the first experiment as a positive control. Twenty-four hours later, all cells were collected using Cell Dissociation Buffer (Thermo Fisher Scientific, Waltham, MA), blocked with anti-Fc antibody, and stained with APC anti-mouse CD11c, PerCP/Cy5.5 anti-mouse MHC Class II (MHCII), Brilliant Violet (BV) 605 anti-mouse CD86 (antibodies purchased from BD Biosciences, Franklin Lakes, NJ), and LIVE/DEAD® Fixable Violet Dead Cell Stain (Thermo Fisher Scientific). After fixation with 1.6% paraformaldehyde, cells were analyzed by flow cytometry (FACSCanto II). Flow cytometry data were analyzed by FlowJo software for surface marker expression of the live, CD11c⁺ gated population.

2.3 Antigen uptake

Cells were seeded at varying CD11c enriched to CD11c reduced isolate ratios in 24-well plates as described above and fluorescently labeled ovalbumin (OVA-Alexa Fluor 647) (Thermo Fisher Scientific) was added to cultures at a concentration of 10 μ g/mL simultaneously with MPLA stimulus (10 μ g/mL). Twenty-four hours later, all cells were collected, washed extensively, stained with PerCP anti-mouse CD11c and LIVE/DEAD® Fixable Violet, fixed with 1.6% paraformaldehyde, and analyzed by flow cytometry (LSRII). Flow cytometry data were analyzed by FlowJo software for OVA-Alexa Fluor 647 signal of live cells.

2.4 Antigen-specific CD4⁺ and CD8⁺ T cell proliferation assay

Following CD11c isolation of bone marrow cells, cells were seeded at CD11c enriched to CD11c reduced isolate ratios of 8:1, 1:1, and 1:8 in 96-well plates at 10,000 total cells/well. Controls of CD11c enriched and reduced cells only were included. Cells were incubated with MPLA stimulus (2 μ g) and EndoFit ovalbumin (20 μ g) (InvivoGen) for 24 hours before

co-culture with T cells. CD11c enriched cells without treatment with ovalbumin were included as a negative control and CD11c enriched cells treated with LPS (0.2 µg) were included as a positive control. CD4⁺ and CD8⁺ T cells were collected from the spleens of OTII and OTI transgenic mice (Jackson Laboratory, New Harbor, ME), respectively. Briefly, spleens were collected and placed in 4 mL digestion media at 1.5 mg/mL Collagenase D and 40 µg/mL DNase I (Sigma-Aldrich, St. Louis, MO) in RPMI-1640. Spleens were then incubated at 37 °C for 30 minutes. Connective tissue was filtered out by a 70 µm cell strainer, and red blood cells were lysed. To select for CD4⁺ or CD8⁺ T cells, remaining splenocytes were incubated with magnetically-labeled antibodies and isolated by negative selection through a magnetic column (Miltenyi). Purified T cells were then labeled with carboxyfluorescein succinimidyl ester (CFSE) (10 µM) (Thermo Fisher Scientific) for proliferation tracking. T cells were co-cultured with OVA- and MPLA-treated CD11c^{+/−} cells at CD11c^{+/−} cells:T cells 1:10 for CD4⁺ and 1:30 for CD8⁺ T cell proliferation analysis. After 72 hours, cells were collected, stained with APC anti-mouse CD4 or APC anti-mouse CD8 (BD Biosciences) and LIVE/DEAD® Fixable Violet, fixed with 1.6% paraformaldehyde, and analyzed by flow cytometry (LSRII). Fraction diluted was quantified by the CFSE signal dilution of the live, CD4⁺ or CD8⁺ population. Proliferation index was calculated using FlowJo v9 Proliferation Platform.

2.5 Analysis of cytokine production

Concentration of cytokines IL-12, IL-10, IL-1β, TNF-α in the cell culture supernatant of CD11c^{+/−} co-cultures after 24 hour MPLA treatment and of cytokines IL-4 and IFN-γ accumulated in the cell culture supernatant from CD11c^{+/−} and T cell cultures after 72 hours were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (PeproTech).

2.6 Statistical analysis

Results are expressed as the mean ± standard deviation. When comparing outcomes between groups, statistical analysis was performed by one-way analysis of variance (ANOVA) using Tukey's multiple comparison post-test. When comparing outcomes between the same group of different conditions (no stimulus vs MPLA-treated), multiple t-tests were used with multiple comparison correction using the Holm-Sidak method. Statistical analyses were performed using GraphPad Prism 6 software. Statistical significance was defined as $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$).

3. Results

3.1 Co-cultures of CD11c⁺ and CD11c[−] cells modulate adjuvant-induced DC maturation

We examined DC maturation marker expression from co-cultures of varying ratios of CD11c⁺ and CD11c[−] cells to understand how these heterogeneous cell populations respond to TLR stimulation. The co-culture groups investigated were CD11c enriched, CD11c reduced, and combinations of 8:1, 1:1, and 1:8, respectively. MPLA-stimulation induced the upregulation of the maturation markers CD86 and MHCII among the CD11c⁺ DC population for all co-culture conditions as quantified by mean fluorescence intensity (MFI). Compared to non-stimulated cells of the same culture condition, stimulation by MPLA resulted in an MFI

increase of up to 14- and 3-fold for CD86 and MHCII respectively, demonstrating the ability of DCs to strongly respond to maturation stimuli (Fig. 1a). However, although all cultures were exposed to the same amount of adjuvant, DCs in the presence of CD11c⁻ cells did not upregulate surface markers CD86 and MHCII to the same extent as when cultured as a CD11c⁺ enriched population (Fig. 1b,c, respectively). Surface upregulation was further dampened with increased fraction of CD11c⁻ cells in the culture. In the absence of MPLA stimulation, the majority of CD11c⁺ cells did not co-express the maturation markers MHCII and CD86 for all co-cultures (Fig. 2a,b). We observed a slight decrease in the percentage of CD11c⁺ cells that were double positive for both markers as the percentage of CD11c⁻ cells in the culture increased, although this trend was not significant. Upon MPLA stimulation, we observed a significantly lower percentage of CD86 and MHCII double positive CD11c⁺ cells in co-cultures containing a higher fraction of CD11c⁻ cells (Fig. 2a,c). Low expression of these activation markers was observed on the CD11c⁻ population for all culture ratios after MPLA stimulation, in which MFI values of CD86 and MHCII were up to ~12- and 9-fold less than that of the CD11c⁺ population, respectively (data not shown). From these data, we can conclude that the presence of CD11c⁻ cells during DC priming with a TLR adjuvant impede MPLA-driven DC maturation and result in DCs with a less mature phenotype.

To investigate how the co-culture conditions modulate cytokine secretion from cells, we analyzed co-culture supernatants both with and without MPLA treatment for the presence of common inflammatory cytokines. Without adjuvant stimulation, all culture conditions secreted low levels of the cytokines IL-12 and TNF- α (Fig. 3a,b), whereas no secretion of IL-10 or IL-1 β was detected. After addition of MPLA, we observed a dramatic increase up to 400- and 56-fold in the concentration of IL-12 and TNF- α , respectively, compared to non-stimulated cells of the same culture condition (Fig. 3a,b). Similar concentrations of TNF- α was detected from all cultures and although not statistically significant, we observed a trend for increased IL-12 secretion with increased percentage of CD11c⁺ cells present. Thus, for the common cytokines investigated, co-culture ratio does not play a role in modulating cytokine secretion.

3.2 Combination of both CD11c⁺ and CD11c⁻ cells enhances antigen uptake by DCs

Given that DC maturation status was observed to be dependent on CD11c⁺/⁻ co-culture ratio, we hypothesized that antigen uptake by MPLA-stimulated CD11c⁺ cells would also be influenced by the presence of CD11c⁻ cells in culture since antigen uptake capacity is downregulated upon DC maturation. The elucidation of this parameter is critical in order to achieve high DC loading of antigen during *ex vivo* priming. Using an OVA-Alexa Fluor 647 antigen, we treated CD11c⁺/⁻ cultures with both MPLA and OVA simultaneously and observed that almost all of the CD11c⁺ population in co-culture with CD11c⁻ cells was OVA⁺. Degree of antigen uptake was found to be dependent on the CD11c⁻ fraction in the culture, as OVA MFI of CD11c⁺ cells increased as the percentage of CD11c⁻ cells in the culture increased (Fig. 4a). In particular, we observed a significant increase in uptake of OVA by almost two-fold in the 1:8 co-culture compared to the CD11c⁺ enriched population. In contrast, a low percentage of the CD11c⁻ population was OVA⁺ and displayed lower OVA signal per cell (Fig. 4b). In summary, the presence of CD11c⁻ cells during DC maturation and antigen loading enhance uptake of antigen in CD11c⁺ DCs.

3.3 Presence of CD11c⁺ cells enhance CD8⁺ T cell proliferation and activation

We next sought to understand the functional differences of DCs when cultured in the presence of CD11c⁺ cells at varying ratios through investigation of CD4⁺ and CD8⁺ T cell proliferation. Through the utilization of T cells bearing transgenic receptors specific for OVA epitopes, we elucidated DC antigen presentation and capacity to stimulate antigen-specific T cells. Upon culture of CD4⁺ T cells with OVA-pulsed, MPLA-stimulated CD11c⁺/⁻ cell ratios, we observed a similar percentage of CD4⁺ T cells that underwent at least one round of proliferation (fraction diluted) from all co-culture conditions, with around 80% of all T cells undergoing at least one round of proliferation (Fig. 5a,b). We also investigated the secretion of T cell cytokines IFN- γ and IL-4 after 72 hours to identify T cell polarization into a T_H1 or T_H2 phenotype, respectively. No IL-4 was detected in any cultures and IFN- γ was detected in all cultures at similar levels (Fig. 5c). These data indicate that the presence of CD11c⁺ cells do not prevent or reduce MHCII presentation to CD4⁺ T cells nor their polarization toward the T_H1 lineage.

Upon co-culture of OVA-pulsed, MPLA-stimulated CD11c⁺/⁻ cells with CD8⁺ T cells, we observed a trend in the percentage of CD8⁺ T cells that underwent at least one round of division and percentage of CD11c⁺ cells in the co-culture (Fig. 6a,b). Strikingly, as the percentage of CD11c⁺ fraction increased in the culture, more rounds of division by CD8⁺ T cells were induced, resulting in an increased proliferation index (i.e. the average number of cell divisions that the responding T cells underwent) of up to 127% (Fig. 6c). The average percentage of CD8⁺ T cells that underwent more than 1 round of proliferation was 15% when cultured with CD11c⁺ enriched cells and increased to 38% when cultured with CD11c⁺ reduced cells. This same trend was observed for the secretion of IFN- γ , in which higher production of the anti-viral cytokine was achieved through co-culture of CD8⁺ T cells with both CD11c⁺ and CD11c⁺ cells (Fig. 6d). These results demonstrate that the presence of CD11c⁺ cells enhance MHCI cross-presentation by antigen-presenting cells and the activation of CD8⁺ T cells compared to cultures of CD11c⁺ only.

4. Discussion and Conclusions

Dendritic cell based immunotherapies have promise in vaccination and therapeutic interventions through the role of DCs to induce antigen-specific adaptive immune responses. [30–32] The importance of the maturation state of the adoptively transferred DC and the types of immune cells present during *ex vivo* priming is known to influence immune responses *in vivo*. For example, the use of the CD11c⁺ immune cell population along with DCs as well as the use of different DC subsets in combination has shown to be beneficial in pre-clinical studies of DC-therapies.[17,22,33,34] Clearly, understanding the *ex vivo* preparation of DCs for immunotherapies can have large impacts on the quality of effector adaptive immunity and the clinical efficacy of such interventions.[5,35] Here, we show for the first time the influence of CD11c⁺ cells at varying percentages in culture upon TLR stimulation on CD11c⁺ DC maturation status, antigen uptake, and ability to activate effector T cells.

In our study, we produced a cell co-culture environment wherein crosstalk can occur among bone marrow-derived CD11c⁺ and CD11c⁺ cells either directly (via cell-cell interaction) or

indirectly (via signaling proteins). We found that the presence of CD11c⁻ cells in culture during adjuvant stimulation resulted in significantly reduced expression of the surface markers CD86 and MHCII of CD11c⁺ cells in a CD11c⁻ dose-dependent manner. According to a previous study, DC derivation from bone marrow results in a population comprised of DCs, macrophages, monocytes, and granulocytes such as neutrophils. These innate immune cells have been shown to communicate with each other both directly and indirectly, ultimately modulating the resulting immune response of the host.[28] Neutrophils can directly communicate with DCs, such as through the binding of DC-SIGN surface receptor, which can induce DC activation. However, ligation of DC-SIGN by neutrophils has also been shown to interrupt TLR 4-mediated signaling of DCs.[36–38] Macrophages also contribute to immune signaling and respond to various stimuli by secreting factors that balance pro-inflammatory (IL-1, IL-6, IL-23) and anti-inflammatory/wound healing (IL-10, TGF- β , IL-4) responses [39,40]. Additionally, regulatory macrophages can inhibit inflammatory functions in response to stress.[39] In these ways, DC phenotype can be modulated by the response of CD11c⁻ cells that are in close proximity, such as in our co-culture system in the present study. Though we investigated DC maturation using cultures of cells derived from monocyte precursors in the bone marrow, for practical clinical application DCs are commonly derived from blood monocytes. While similar cell types may be generated through both methods, the specific effect of non-DCs from protocols using blood monocytes will need to be investigated.

Robust uptake of antigen by DCs is critical in *ex vivo* DC preparation for enhancing *in vivo* stimulation of T cells.[41,42] In accordance with down-regulation of antigen endocytosis of DCs upon maturation [43–45], we found that OVA uptake positively correlated with immature status of CD11c⁺ cells in the co-culture. However, due to the nature of our experimental set-up, the amount of OVA increased per CD11c⁺ cell in co-cultures with increased CD11c⁻ cells. Thus, we do not know how the combination of the more immature status of CD11c⁺ cells and the increased ratio of OVA to CD11c⁺ cell contributed to OVA uptake in the CD11c⁺ population. Interestingly, we also found that OVA was uptaken primarily by CD11c⁺ cells in the co-culture system. This uptake peaked at the co-culture ratio of 1:8 but not the CD11c reduced control, which included CD11c⁺ cells that exhibited the lowest levels of maturation phenotype. Therefore, it is conceivable that the immature status of CD11c⁺ DCs still positively controls antigen uptake but there is specific ratio of antigen concentration to a single CD11c⁺ cell for a maximum antigen uptake. These indicate that we can possibly generate DCs using the co-culture system with both desired qualities of maximum antigen loading and mature phenotype.[35,46,47]

Ability of DCs to stimulate CD4⁺ and CD8⁺ T cells ultimately controls the effector functionality of the therapy. For applications in DC-based cancer immunotherapy, the elicitation of both CD8⁺ cytotoxic T lymphocytes (CTLs) and T_H1 CD4⁺ T cells is critical for tumor eradication.[34] The generation of CTLs requires the presentation of exogenous antigens or proteins onto MHC Class I. DCs possess this function through adapting their endocytic and phagocytic pathways.[48] Cross-presentation of extracellular peptide onto MHCI molecules have been shown to be favored for high density antigens.[49] Thus, if more OVA is being taken up by CD11c⁺ cells when co-cultured with CD11c⁻ cells during MPLA stimulation and antigen priming, it is possible that there is higher frequency of OVA-

MHCI complexes that are able to activate CD8⁺ T cells. Additionally, depending on the maturation state of the DC, encountered antigens can be favored to be processed in distinct endocytic compartments, which can influence the fate of the antigen for MHC I or MHC II presentation.[50] Thus, OVA presentation on MHC I may be favored when cells are cultured with both CD11c⁺ and CD11c⁻ cells, which is supported by our data. These finding may contribute to the results of a previous study, in which there was a significant increase in the percentage of tumor-free animals when vaccinated with both CD11c⁺ and CD11c⁻ cells but not with CD11c⁺ cells alone.[51]

In our study we expected highest CD4⁺ T cell proliferation from the CD11c⁺ isolate only culture, in which we observed a CD11c⁺ cell phenotype of higher frequencies and densities of the costimulatory molecules MHC II and CD86. However, as we observed similar abilities of all co-cultures to activate CD4⁺ T cells and induce IFN- γ secretion (T_H1 polarization), our results may indicate that the maturation level of the DCs, the number of mature DCs, or both was sufficient to activate CD4⁺ T cells from all culture ratios. Importantly, the presence of CD11c⁻ cells did not hinder the ability of DCs to present to and activate CD4⁺ T cells. Results from both CD8⁺ and CD4⁺ T cell proliferation studies highlight the phenotypic differences in cells derived using different culture methods that are important to keep in mind for future studies, in which certain types of immune responses are desired for therapeutic efficacy.

In conclusion, we have shown that by varying the cellular composition in culture during DC priming and maturation, adaptive immune responses can be modulated, most notably amplifying CD8⁺ T cell stimulation and the production of type II interferons. Our data thus support the use of CD11c⁺ DCs and other immune cell populations, such as monocytes and granulocytes, as an *ex vivo* DC culture strategy for DC-therapies to activate CD4⁺ T cells while enhancing the expansion of CD8⁺ T cells, which are critical for the success of immunotherapies. Future work should focus on mechanisms of communication between heterogeneous CD11c⁺ and CD11c⁻ populations and on recapitulation of this observed increase in cross-priming of CD8⁺ T cells in *in vivo* experiments.

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Abbreviations

DC	Dendritic cell
GM-CSF	granulocyte-macrophage colony stimulating factor
TLR	Toll-like receptor
MPLA	monophosphoryl lipid A
LPS	lipopolysaccharide

T_H1	T-helper 1
T_H2	T-helper 2
OVA	ovalbumin
CFSE	carboxyfluorescein succinimidyl ester
MFI	mean fluorescence intensity
CTL	cytotoxic T lymphocyte

References

1. Guernonprez P, Valladeau J, Zitvogel L, Théry C, Amigorena S. Antigen Presentation and T Cell Stimulation by Dendritic Cells. *Annu Rev Immunol.* 2002; 20:621–667. DOI: 10.1146/annurev.immunol.20.100301.064828 [PubMed: 11861614]
2. Figdor CG, de Vries IJM, Lesterhuis WJ, Melief CJM. Dendritic cell immunotherapy: mapping the way. *Nat Med.* 2004; 10:475–80. DOI: 10.1038/nm1039 [PubMed: 15122249]
3. Palucka K, Banchereau J. Dendritic-Cell-Based Therapeutic Cancer Vaccines. *Immunity.* 2013; doi: 10.1016/j.immuni.2013.07.004
4. Kazemi T, Younesi V, Jadidi-Niaragh F, Yousefi M. Immunotherapeutic approaches for cancer therapy: An updated review Tumor escape from immune system. *Artif Cells, Nanomedicine, Biotechnol.* 2015; :1–11. DOI: 10.3109/21691401.2015.1019669
5. Chiang CLL, Balint K, Coukos G, Kandalaft LE. Potential approaches for more successful dendritic cell-based immunotherapy. *Expert Opin Biol Ther.* 2015; 15:569–582. DOI: 10.1517/14712598.2015.1000298 [PubMed: 25553913]
6. Bonaccorsi I, Pezzino G, Morandi B, Ferlazzo G. Novel perspectives on dendritic cell-based immunotherapy of cancer. *Immunol Lett.* 2013; 155:6–10. DOI: 10.1016/j.imlet.2013.09.021 [PubMed: 24076312]
7. Neill DWO, Adams S, Bhardwaj N. Review in translational hematology Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood J.* 2015; 104:2235–2247. DOI: 10.1182/blood-2003-12-4392.Supported
8. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. *Nat Immunol.* 2001; doi: 10.1038/ni712
9. Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. *Vaccine.* 2011; doi: 10.1016/j.vaccine.2010.08.002
10. Pasare C, Medzhitov R, Haven N. Toll-Dependent Control Mechanisms of CD4 T Cell Activation. 2004; 21:733–741.
11. De Becker G. The adjuvant monophosphoryl lipid A increases the function of antigen-presenting cells. *Int Immunol.* 2000; 12:807–815. DOI: 10.1093/intimm/12.6.807 [PubMed: 10837408]
12. Ismaili J, Rennesson J, Aksoy E, Vekemans J, Vincart B, Amraoui Z, Van Laethem F, Goldman M, Dubois PM. Monophosphoryl lipid A activates both human dendritic cells and T cells. *J Immunol.* 2002; 168:926–932. DOI: 10.4049/jimmunol.168.2.926 [PubMed: 11777991]
13. Draube A, Klein-González N, Mattheus S, Brilliant C, Hellmich M, Engert A, von Bergwelt-Baildon M. Dendritic cell based tumor vaccination in prostate and renal cell cancer: A systematic review and meta-analysis. *PLoS One.* 2011; 6doi: 10.1371/journal.pone.0018801
14. Jonuleit H, Giesecke-Tuettenberg A, Tüting T, Thurner-Schuler B, Stuge TB, Paragnik L, Kandemir A, Lee PP, Schuler G, Knop J, Enk AH. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int J Cancer.* 2001; 93:243–251. DOI: 10.1002/ijc.1323 [PubMed: 11410873]
15. Labeur MS, Roters B, Pers B, Mehling A, Luger TA, Schwarz T, Grabbe S. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J*

- Immunol. 1999; 162:168–75. [accessed January 5, 2016] <http://www.jimmunol.org/content/162/1/168.full>. [PubMed: 9886383]
16. Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzzi C, Falo LD, Melief CJ, Ildstad ST, Martin Kast W, Deleo AB, Lotze MT. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat Med.* 1995; 1:1297–1302. DOI: 10.1038/nm1295-1297 [PubMed: 7489412]
 17. Campisano S, Mac Keon S, Gazzaniga S, Ruiz MS, Traian MD, Mordoh J, Wainstok R. Anti-melanoma vaccinal capacity of CD11c–positive and -negative cell populations present in GM-CSF cultures derived from murine bone marrow precursors. *Vaccine.* 2013; doi: 10.1016/j.vaccine.2012.10.114
 18. Mendoza L, Bubeník J, Símová J, Jandlová T, Vonka V, Mikysková R. Prophylactic, therapeutic and anti-metastatic effects of BMDC and DC lines in mice carrying HPV 16-associated tumours. *Int J Oncol.* 2003; 23:243–247. [PubMed: 12792800]
 19. Butterfield LH. Dendritic cells in cancer immunotherapy clinical trials: Are we making progress? *Front Immunol.* 2013; 4:3–9. DOI: 10.3389/fimmu.2013.00454 [PubMed: 23346089]
 20. Rescigno M, Lopatin U, Chieppa M. Interactions among dendritic cells, macrophages, and epithelial cells in the gut: implications for immune tolerance. *Curr Opin Immunol.* 2008; 20:669–675. DOI: 10.1016/j.coi.2008.09.007 [PubMed: 18852045]
 21. Diana J, Simoni Y, Furio L, Beaudoin L, Agerberth B, Barrat F, Lehuen A. Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes. *Nat Med.* 2013; doi: 10.1038/nm.3042
 22. Bakdash G, Schreurs I, Schreibelt G, Tel J. Crosstalk between dendritic cell subsets and implications for dendritic cell-based anticancer immunotherapy. *Expert Rev Clin Immunol.* 2014; 10:915–26. DOI: 10.1586/1744666X.2014.912561 [PubMed: 24758519]
 23. Shen H, Iwasaki A. A crucial role for plasmacytoid dendritic cells in antiviral protection by CpG ODN-based vaginal microbicide. *J Clin Invest.* 2006; doi: 10.1172/JCI28681
 24. Kelso A. Cytokines: Principles and prospects. *Immunol Cell Biol.* 1998; 76:300–317. DOI: 10.1046/j.1440-1711.1998.00757.x [PubMed: 9723771]
 25. Schmitz ML, Weber A, Roxlau T, Gaestel M, Kracht M. Signal integration, crosstalk mechanisms and networks in the function of inflammatory cytokines. *Biochim Biophys Acta - Mol Cell Res.* 2011; 1813:2165–2175. DOI: 10.1016/j.bbamcr.2011.06.019
 26. Carroll-Portillo A, Cannon JL, Te Riet J, Holmes A, Kawakami Y, Kawakami T, Cambi A, Lidke DS. Mast cells and dendritic cells form synapses that facilitate antigen transfer for T cell activation. *J Cell Biol.* 2015; 210:851–864. DOI: 10.1083/jcb.201412074 [PubMed: 26304724]
 27. Striz I, Slavcev a, Kalanin J, Jaresová M, Rennard SI. Cell-cell contacts with epithelial cells modulate the phenotype of human macrophages. *Inflammation.* 2001; 25:241–246. DOI: 10.1023/A:1010975804179 [PubMed: 11580100]
 28. Rivera A, Siracusa MC, Yap GS, Gause WC. Innate cell communication kick-starts pathogen-specific immunity. *Nat Immunol.* 2016; 17:356–363. DOI: 10.1038/ni.3375 [PubMed: 27002843]
 29. Lutz MB, Kukutsch N, Ogilvie aL, Rössner S, Koch F, Romani N, Schuler G. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999; 223:77–92. DOI: 10.1016/S0022-1759(98)00204-X [PubMed: 10037236]
 30. Anguille S, Smits EL, Lion E, Van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. *Lancet Oncol.* 2014; 15:257–267. DOI: 10.1016/S1470-2045(13)70585-0 [PubMed: 24534291]
 31. Datta J, Terhune JH, Lowenfeld L, Cintolo Ja, Xu S, Roses RE, Czerniecki BJ. Optimizing dendritic cell-based approaches for cancer immunotherapy. *Yale J Biol Med.* 2014; 87:491–518. DOI: 10.1586/14760584.6.3.333 [PubMed: 25506283]
 32. Bol KF, Schreibelt G, Gerritsen WR, de Vries IJM, Figdor CG. Dendritic Cell-Based Immunotherapy: State of the Art and Beyond. *Clin Cancer Res.* 2016; 22:1897–1906. DOI: 10.1158/1078-0432.CCR-15-1399 [PubMed: 27084743]
 33. Wimmers F, Schreibelt G, SkÅ¶ld AE, Figdor CG, De Vries IJM. Paradigm Shift in Dendritic Cell-Based Immunotherapy: From in vitro Generated Monocyte-Derived DCs to Naturally Circulating

- DC Subsets. *Front Immunol.* 2014; 5:1–12. DOI: 10.3389/fimmu.2014.00165 [PubMed: 24474949]
34. Sittig SP, Bakdash G, Weiden J, Sköld AE, Tel J, Figdor CG, De Vries IJM, Schreiber G. A Comparative Study of the T Cell Stimulatory and Polarizing Capacity of Human Primary Blood Dendritic Cell Subsets. *Mediators Inflamm.* 2016; 2016doi: 10.1155/2016/3605643
 35. Dewitte H, Verbeke R, Breckpot K, Vandenbroucke RE, Libert C, De Smedt SC, Lentacker I. Choose your models wisely: how different murine bone marrow-derived dendritic cell protocols influence the success of nanoparticulate vaccines in vitro. *J Control Release.* 2014; 195:138–46. DOI: 10.1016/j.jconrel.2014.06.024 [PubMed: 24960224]
 36. van Gisbergen KPJM. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med.* 2005; 201:1281–1292. DOI: 10.1084/jem.20041276 [PubMed: 15837813]
 37. Ludwig IS, Geijtenbeek TBH, van Kooyk Y. Two way communication between neutrophils and dendritic cells. *Curr Opin Pharmacol.* 2006; 6:408–13. DOI: 10.1016/j.coph.2006.03.009 [PubMed: 16750420]
 38. Geijtenbeek TBH, van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CMJE, Appelmek B, van Kooyk Y. Mycobacteria Target DC-SIGN to Suppress Dendritic Cell Function. *J Exp Med.* 2002; 197:7–17. DOI: 10.1084/jem.20021229
 39. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 2008; 8:958–69. DOI: 10.1038/nri2448 [PubMed: 19029990]
 40. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 2014; 6:13.doi: 10.12703/P6-13 [PubMed: 24669294]
 41. Bonifaz LC, Bonnyay DP, Charalambous A, Darguste DI, Fujii SI, Soares H, Brimnes MK, Moltedo B, Moran TM, Steinman RM. In Vivo Targeting of Antigens to Maturing Dendritic Cells via the DEC-205 Receptor Improves T Cell Vaccination. *J Exp Med J Exp Med.* 2004; 038151000:815–824. DOI: 10.1084/jem.20032220
 42. Strome SE, Voss S, Wilcox R, Wakefield TL, Tamada K, Flies D, Chapoval A, Lu J, Kasperbauer JL, Padley D, Vile R, Gastineau D, Wettstein P, Chen L. Strategies for Antigen Loading of Dendritic Cells to Enhance the Antitumor Immune Response Strategies for Antigen Loading of Dendritic Cells to Enhance the Antitumor. 2002:1884–1889.
 43. Steinman BRM, Steinman BRM, Swanson J, Swanson J. The Endocytic Activity of Dendritic Cells. *Commentary.* 1995; 182
 44. Mellman I, Steinman RM. Dendritic Cells: Specialized and Regulated Antigen Processing Machines. *Cell.* 2001; 106:255–258. [http://dx.doi.org/10.1016/S0092-8674\(01\)00449-4](http://dx.doi.org/10.1016/S0092-8674(01)00449-4). [PubMed: 11509172]
 45. Blander JM. Phagocytosis and antigen presentation: a partnership initiated by Toll-like receptors. *Ann Rheum Dis.* 2008; 67(Suppl 3):iii44–i49. DOI: 10.1136/ard.2008.097964 [PubMed: 19022813]
 46. Lanzavecchia, a, Sallusto, F. Regulation of T cell immunity by dendritic cells. *Cell.* 2001; 106:263–266. DOI: 10.1016/S0092-8674(01)00455-X [PubMed: 11509174]
 47. Reschner A, Hubert P, Delvenne P, Boniver J, Jacobs N. Innate lymphocyte and dendritic cell cross-talk: A key factor in the regulation of the immune response. *Clin Exp Immunol.* 2008; 152:219–226. DOI: 10.1111/j.1365-2249.2008.03624.x [PubMed: 18336590]
 48. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol.* 2012; 12:557–569. DOI: 10.1038/nri3254 [PubMed: 22790179]
 49. Kurts C, Miller JF, Subramaniam RM, Carbone FR, Heath WR. Major histocompatibility complex class I-restricted cross-presentation is biased towards high dose antigens and those released during cellular destruction. *J Exp Med.* 1998; 188:409–414. DOI: 10.1084/jem.188.2.409 [PubMed: 9670054]
 50. Chatterjee B, Smed-Sörensen A, Cohn L, Chalouni C, Vandlen R, Lee BC, Widger J, Keler T, Delamarre L, Mellman I. Internalization and endosomal degradation of receptor-bound antigens regulate the efficiency of cross presentation by human dendritic cells. *Blood.* 2012; 120:2011–2020. DOI: 10.1182/blood-2012-01-402370 [PubMed: 22791285]

51. Campisano S, Mac Keon S, Gazzaniga S, Ruiz MS, Traian MD, Mordoh J, Wainstok R. Anti-melanoma vaccinal capacity of CD11c– positive and -negative cell populations present in GM-CSF cultures derived from murine bone marrow precursors. *Vaccine*. 2013; 31:354–61. DOI: 10.1016/j.vaccine.2012.10.114 [PubMed: 23146677]

Highlights

- Co-culture with CD11c⁺ cells reduced TLR4-induced DC maturation.
- DC antigen uptake enhanced through co-culture with CD11c⁺ cells.
- Presence of CD11c⁺ cells during priming did not reduce CD4⁺ T cell proliferation.
- CD8⁺ T cell proliferation enhanced by DC co-culture with CD11c⁺ cells.

Figure 1. Presence of bone marrow-derived CD11c⁺ cells reduces extent of inflammatory surface marker expression by CD11c⁺ DCs upon MPLA stimulation

Bone marrow-derived cells were separated into CD11c enriched and CD11c reduced isolates, co-cultured at varying ratios, and treated with or without MPLA (10 μ g/mL). (a) The CD11c⁺ population was analyzed for expression of CD86 and MHCII. Relative mean fluorescence intensity (MFI) of (b) CD86 and (c) MHCII of CD11c⁺ cells after MPLA stimulation for 24 hours under different co-culture conditions. Untreated and LPS-treated (1 μ g/mL) CD11c enriched cells were included as negative and positive controls, respectively. Data is representative of the mean of three independent experiments $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance using Tukey's multiple comparison post-test comparing groups against the CD11c enriched culture condition. *** p <0.005; ** p <0.01; * p <0.05.

Figure 2. Presence of bone marrow-derived CD11c⁻ cells reduces CD11c⁺ DC maturation by MPLA adjuvant

Bone marrow-derived cells were separated into CD11c enriched and CD11c reduced isolates, co-cultured at varying ratios, and treated with or without MPLA (10 $\mu\text{g/mL}$). (a) Representative flow plots showing CD86 and MHCII co-expression on CD11c⁺ cells both with and without MPLA stimulation. Percentage of live, CD11c⁺ cells that were CD86⁺MHCII⁺ double positive 24 hours after co-culture at varying ratios (b) without MPLA stimulation and (c) with MPLA stimulation. LPS-treated (1 $\mu\text{g/mL}$) CD11c enriched cells were included as a positive control. Data is representative of the mean of three independent experiments $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance using Tukey's multiple comparison post-test comparing groups against the CD11c enriched culture condition. ** $p < 0.01$; * $p < 0.05$.

Figure 3. MPLA adjuvant stimulation induces enhanced inflammatory cytokine expression by all CD11c+/- co-culture ratios

Bone marrow-derived cells were separated into CD11 enriched and CD11c reduced isolates, co-cultured at varying ratios, and received either no stimulus or MPLA treatment (10 µg/mL). Supernatants were assessed by ELISA for the presence of (a) interleukin-12 (IL-12) and (b) tumor necrosis factor alpha (TNF-α) after 24 hours. Data is representative of the mean of three independent experiments ± standard deviation. Statistical analysis was performed by comparing no stimulus and MPLA treatment values for each co-culture condition using multiple t-tests. ****p<0.001; ***p<0.005.

Figure 4. Presence of CD11c⁻ cells enhances antigen uptake in CD11c⁺ DCs

Bone marrow-derived cells were separated into CD11c enriched and CD11c reduced isolates, co-cultured at varying ratios, and were incubated with ovalbumin-Alexa Fluor 647 (OVA) (10 μ g) in combination with MPLA treatment (10 μ g/mL). The presence of OVA was analyzed by flow cytometry for both the (a) CD11c⁺ population and (b) CD11c⁻ population. CD11c enriched cells not treated with adjuvant were included as a negative control. To reduce mouse-to-mouse variability, OVA MFI was normalized to the CD11c⁺ population from the CD11c enriched culture condition from each experimental replicate. Data is representative of the mean of three independent experiments $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance using Tukey's multiple comparison post-test comparing groups against the CD11c enriched culture condition. * $p < 0.05$.

Figure 5. Equivalent activation of antigen-specific CD4⁺ T cells by all CD11c^{+/-} co-culture ratios
 Bone marrow-derived cells were separated into CD11c enriched and CD11c reduced isolates, co-cultured at varying ratios, incubated with ovalbumin (OVA) (10 μ g) in combination with MPLA treatment (10 μ g/mL) for 24 hours, and then cultured with OVA-specific CFSE⁺ CD4⁺ T cells at CD11c^{+/-}:T cell 1:10 for 72 hours. (a) Dilution of CFSE⁺ signal among CD4⁺ T cells after culture with CD11c^{+/-} cells was observed by flow cytometry. (b) Fraction of CD4⁺ T cells diluted with respect to CD11c^{+/-} ratio. (c) Supernatants were assessed by ELISA for the presence of interferon gamma (IFN- γ). CD11c enriched cells that were not incubated with ovalbumin antigen before co-culture with T cells were included as a negative control for the assay. Untreated and LPS-treated (0.2 μ g) CD11c enriched cells were included as negative and positive controls, respectively. Data is representative of the mean of three independent experiments $\pm$ standard deviation.

Figure 6. Presence of CD11c+ cells enhances antigen-specific CD8+ T cell priming

Bone marrow-derived cells were separated into CD11c enriched and CD11c reduced isolates, co-cultured at varying ratios, incubated with ovalbumin (OVA) (10 μ g) in combination with MPLA treatment (10 μ g/mL) for 24 hours, and then cultured with OVA-specific CFSE+ CD8+ T cells at CD11c+/-:T cell 1:30 for 72 hours. (a) Dilution of CFSE+ signal among CD8+ T cells after culture with CD11c+/- cells was observed by flow cytometry. (b) Fraction of CD8+ T cells diluted and (c) proliferation index of CD8+ T cells with respect to CD11c+/- ratio. (d) Supernatants were assessed by ELISA for the presence of interferon gamma (IFN- γ). CD11c enriched cells that were not incubated with ovalbumin antigen before co-culture with T cells were included as a negative control for the assay. Untreated and LPS-treated (0.2 μ g) CD11c enriched cells were included as negative and positive controls, respectively. Data is representative of the mean of two independent experiments $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance using Tukey's multiple comparison post-test comparing groups against the CD11c enriched culture condition. * p <0.05.