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Comparing macrophage differentiation from monocytes isolated via negative or positive magnetic bead selection and performance in an immunotoxicity assay

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Macrophages play a key regulatory role in driving initial inflammatory responses to infections, wounds and toxins.[1] Cytokine signaling at the sites of wounds or infections triggers circulating monocytes to differentiate into naïve macrophages, which polarize into pro-inflammatory M1 macrophages or anti-inflammatory (reparative) M2 macrophages.[2] Replicating the process of generating macrophages *in vitro* requires a delicate balance of many factors across monocyte isolation and macrophage differentiation protocols.[2-3] In order to establish cell culture models that accurately produce the desired subtypes of macrophages, it is important to understand the factors that impact macrophage subtype and physiology. In this study, we examined if isolating $CD14^+$ monocytes via negative selection (NS $CD14^+$) or positive selection (PS $CD14^+$) from human peripheral blood mononuclear cells using magnetic beads affected the phenotypes of differentiated naïve and polarized M1 and M2 macrophages as well as the phagocytic capacity of each subtype with and without an immunotoxic inhibitor.

Methods

The experimental protocol for working with cryopreserved positive bead-selected monocytes used in this study can be found on the Lonza website.[4] Note that the published protocol includes workflows for two different starting materials (cryopreserved PBMCs or cryopreserved positive bead-selected monocytes). The workflow starting with cryopreserved positive bead-selected monocytes used in this study is also used for negative bead-selected monocytes. Materials required for this protocol are listed in Table 1, and the protocol is summarized below

Supplier	Part no.	Description	Size
Lonza	2W-400A 2W-400B 2W-400C	Cryopreserved Human Peripheral Blood CD14⁺ Monocytes, Positively Selected	≥40 million cells ≥20 million cells ≥10 million cells
	2W-401A 4W-400	Cryopreserved Human Peripheral Blood CD14⁺ Monocytes, Positively Selected	≥10 million cells ≥10 million cells
	MKC-500BK	KuGM™ Kuppfer Cell Culture BulletKit®	500 mL basal medium + supplement kit
Fisher Scientific*	P35366	Invitrogen™ pHrodo™ Green *E.coli* BioParticles™ Conjugate for Phagocytosis	5 + 2 mg vials
MilliporeSigma*	C8273-1MG	Cytochalasin D	1 mg vial
	L-5668	LPS	2 mL vial
PeproTech*	300-02	Recombinant Human IFN-γ	20 µg vial
	200-04	Recombinant Human IL-4	20 µg vial
	200-10	Recombinant Human IL-10	10 µg vial
	300-25	Recombinant Human M-CSF	10 µg vial
Any		24-well tissue culture plates 96-well tissue culture plates	
		PBS without Calcium or Magnesium	
		0.4% Trypan Blue Solution	
		Live Cell Imaging System	

Table 1:
Materials used for monocyte differentiation, macrophage polarization, and phagocytosis assays detailed in this study.

*Other vendors can be substituted; however, this may require re-optimization of the protocol due to potential variations in the potency and composition of materials from other vendors.

Cryopreserved NS CD14⁺ (n=3 donors) or PS CD14⁺ (n=4 donors) monocytes were thawed and counted using trypan blue and a hemocytometer. Cells were then resuspended in a modified KuGM™ Medium (KuGM™ prepared according to instructions except using only 500 µL of the gentamycin-amphotericin supplement) supplemented with M-CSF at a density of 1×10^6 cells/mL and seeded into 24 or 96-well tissue culture plates at densities of 1×10^6 or 0.1×10^6 cells/well, respectively. The 24-well plates were used for assessing macrophage polarization, while the 96-well plates were used in the live-cell imaging phagocytosis assay. Cells were left undisturbed for three days, after which the medium was changed and replaced with fresh modified KuGM™ Medium supplemented with M-CSF and incubated for another three days.

On day 6, macrophage polarization was induced by replacing media with polarization medium consisting of modified KuGM™ Medium with IFN-γ and LPS ("M1 Polarization Medium") or IL-4 and IL-10 ("M2 Polarization Medium"). Control wells with naïve macrophages were maintained with modified KuGM™ Medium supplemented with M-CSF. Cells were incubated for an additional 24 hours to allow for polarization prior to downstream assays.

Monocytes and macrophages were assessed for morphology using phase contrast microscopy and for internal marker expression via FACS analysis. Performance of each macrophage subtype in a phagocytosis assay was quantified through live-cell imaging. This assay assessed the rate at which naïve, M1 and M2 macrophages consumed pHrodo™ labeled *E. coli* bioparticles. On day 7, polarization medium was removed from all wells. Cells were washed with modified KuGM™ Medium, incubated for an hour, and treated with pHrodo™ bioparticles (10 µg/well) with or without 1 µM cytochalasin D (phagocytosis inhibitor) prior to live-cell imaging. Full details of this protocol can be found on the Lonza website.[4]

Results and discussion

Naïve, M1 and M2 macrophages derived from either NS CD14⁺ or PS CD14⁺ cryopreserved monocytes displayed cell morphology consistent with each of the respective macrophage subtypes (Figure 1). Typically, M1 macrophages adopt a more rounded appearance, while both naïve and M2 macrophages exhibit a somewhat elongated appearance.[5] Both naïve and polarized macrophages generally exhibited cell surface and internal marker expression consistent with their respective phenotypes (Table 2, Figure 2), though there was greater donor-to-donor variation among macrophages derived from NS CD14⁺ monocytes. Because of this variability, macrophages derived from NS CD14⁺ monocytes tended to have a more mixed phenotype (Figure 2A) than macrophages derived from PS CD14⁺ monocytes, though no differences were statistically significant. For example, in one donor of naïve macrophages derived from NS CD14⁺ monocytes, expression of CD206 (an M2 phenotypic marker) was very high, suggesting a more anti-inflammatory phenotype that could be driven by the presence of platelets.[3] Interestingly, M2 macrophages derived from NS CD14⁺ monocytes had higher expression of CD80 (an M1 phenotypic marker) primarily driven by one donor, which may not have been related to monocyte selection method. M1 macrophages derived from both NS CD14⁺ and PS CD14⁺ expressed CD80 at near 100%, as expected, but also modest levels of CD206 (Figure 2). Positive magnetic bead isolation has been shown to shift monocyte-derived macrophages towards a more anti-inflammatory, M2-like phenotype.[3] Despite being a marker for M2 macrophages, all monocyte-derived macrophages in this study expressed high levels of CD163 likely due to the use of M-CSF to differentiate monocytes into naïve macrophages.[6,7]

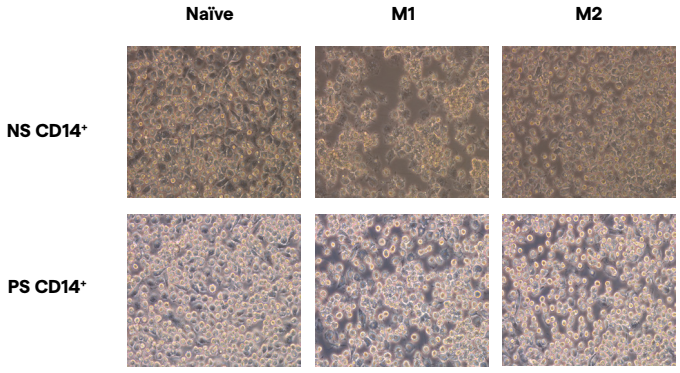

Figure 1:
Representative morphological images from one donor of NS CD14⁺ and PS CD14⁺ monocyte-derived naïve, M1 and M2 macrophages. M1 macrophages should generally have a more rounded appearance, while naïve and M2 macrophages should have a somewhat elongated appearance. Method of monocyte isolation did not have an impact on morphology of any of the macrophage subtypes.

Macrophage cell type	Expected marker expression
Naïve	CD14, CD68
M1	CD14, CD68, CD80
M2	CD14, CD68, CD163, CD206

Table 2:
Expected expression patterns of macrophage surface markers assessed in this study.

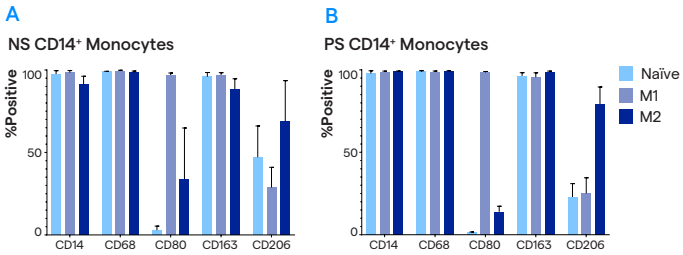

Figure 2:
Percent marker expression for each macrophage subtype derived from cryopreserved NS CD14⁺ (A) and PS CD14⁺ (B) monocytes.

Figure 3 shows phagocytosis assay results for NS CD14⁺ (A) and PS CD14⁺ (B) monocyte-derived macrophages. Naïve and M2 macrophages generally had high rates of phagocytosis, while M1 macrophages had the lowest rates of phagocytosis (Figure 3). In all cases, exposure to cytochalasin D greatly reduced phagocytic activity. As discussed before, bead isolation can shift monocyte populations towards a more M2-like anti-inflammatory phenotype, which could explain why naïve macrophages generally had high rates of phagocytosis, similar to M2 macrophages.[3] The differences in phagocytic activity observed between the M1 macrophages and M2 and naïve macrophages are in line with previous research that shows higher phagocytic activity in M2 macrophages compared to M1 macrophages,[8] and that M2 macrophages phagocytose *E. coli* at a much higher rate than M1 macrophages.[9] This pattern held true for all donors except for one donor of NS CD14⁺ macrophages. In this donor, rates of phagocytosis for naïve macrophages were much lower than M2 macrophages and were much closer to that of the corresponding M1 macrophages (data not shown). Naïve and M2 macrophages in this donor expressed very low levels of CD206, suggesting that there may have been an underlying inflammatory effect that led to naïve macrophages functioning more like M1 macrophages than M2 macrophages. Macrophages are extremely plastic and can exist along a continuum, shifting between M1 and M2-like phenotypes in response to various contexts,[1] so it can be difficult to pinpoint the exact causes of donor-to-donor variation in marker expression and phagocytic activity.

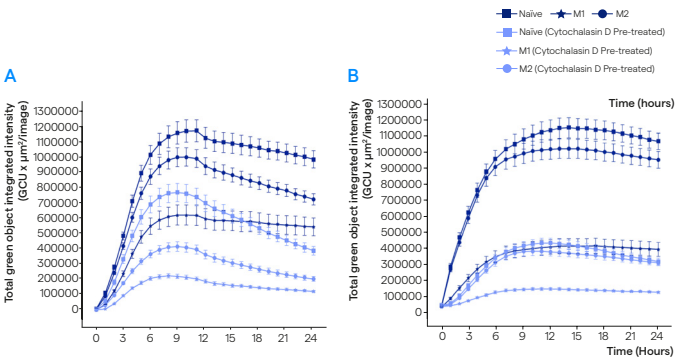

Figure 3:
Results of macrophage phagocytosis assays calculated from real-time fluorescent imagery captured by a live-cell imaging instrument. (A) Macrophages derived from NS CD14⁺ monocytes. (B) Macrophages derived from PS CD14⁺ monocytes. Higher intensity curves indicate greater amounts of phagocytic activity as macrophages actively uptake the *E. coli* bioparticles and trigger fluorescence. Data from single representative donors.

Conclusion

Despite donor-to-donor variation in both marker expression and phagocytic activity in NS CD14⁺ monocyte-derived macrophages, there is little difference between the overall phenotype and functionality of macrophages derived from cryopreserved NS CD14⁺ versus PS CD14⁺ monocytes. Macrophages derived from either type of monocyte exhibit the same inhibitory response to cytochalasin D, demonstrating that using either source of monocyte as a starting point for maturing and differentiating monocytes into macrophage subpopulations is an appropriate choice.

References:

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