Differentiation of CX3CR1⁺ mouse bone marrow cells after magnetic isolation using BioLegend’s MojoSort™ separation system.

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Highlights

- MojoSort™ magnetic cell separation products allowed for depletion of granulocytes and positive enrichment of CX3CR1⁺ precursors from mouse bone marrow.
- CX3CR1⁺ precursors were successfully differentiated in vitro into macrophages or dendritic cells with GM-CSF or GM-CSF and IL-4 respectively.
- Macrophages and dendritic cells expressed a unique cytokine and chemokine profile in response to TLR agonists, as measured with LEGENDplex™.
- The positively enriched CX3CR1⁺ precursors retained in vivo migratory function as demonstrated by their capacity to populate a recipient mouse bone marrow in an adoptive transfer model.
Summary

As bone-marrow derived macrophages and dendritic cells can share several phenotypic markers, it is important for researchers to understand other characteristics of these cell types as they play pivotal roles in several disease states. Using a variety of BioLegend reagents, we implemented a protocol to isolate CX3CR1+ bone marrow cells using magnetic particles, and cultured them under canonical conditions that promote macrophage or dendritic cell differentiation. We then characterized their ability to produce cytokines and chemokines and upregulate costimulatory molecules in response to inflammatory stimuli. In addition, we tested and confirmed the ability of the CX3CR1+ bone marrow precursors to migrate in vivo upon adoptive transfer. Our studies enable researchers to better understand these vital cells and the roles they play in immunity.

Introduction

Chemokines and their receptors constitute a large family of proteins whose main function is to promote cell migration through a concentration gradient. The chemokine molecular signature includes four conserved cysteine residues that form two disulfide bonds pairing the first with the third and the second with the fourth. Based on the arrangement of the N-terminal two cysteine residues, chemokines can be grouped into four subfamilies: CXC, CC, (X)C, and CX3C. CXC chemokines have one amino acid that separates the first two cysteines. In CC chemokines, these two cysteines are adjacent. In the (X)C subfamily, the first and third cysteines are missing. The last subfamily, CX3C, has one member, CX3CL1/fractalkine, and it has three amino acids between the two cysteines.

CX3CR1 is the receptor of CX3CL1 and is a G-protein-coupled, seven-transmembrane domain receptor. It is expressed by innate cells, predominantly macrophages and microglia, but also by other cell types such as T cell subsets. Given the nature of the cells expressing the receptor, CX3CL1-CX3CR1 interaction plays a fundamental role in a number of conditions, from Alzheimer’s1 to atherosclerosis2 and mucosal immunity3.

Although expression of CX3CR1 in circulating and resident populations has been well-studied using a GFP/CX3CR1 reporter mouse model, CX3CR1+ bone marrow precursors are not as well-known. In this report, we assess the capacity of murine CX3CR1+ bone marrow cells to differentiate into macrophages or dendritic cells when cultured with GM-CSF or GM-CSF/IL-4 after positive selection. We show the surface phenotype and cytokine and chemokine profile of TLR-stimulated cells, as well as their capacity to migrate after in vivo transfer.
Materials and Methods

Bone marrow single cell suspensions
There are several published methods to obtain a single cell suspension from mouse bone marrow. Briefly, 8-12 weeks old C57BL/6 mice were sacrificed, femurs were dissected, and excess tissue was removed under aseptic conditions. Bones were rinsed and transferred to complete media. The ends of the bone were trimmed with sterilized scissors to expose the marrow. Marrow was flushed using a syringe and a 29G x ½ needle. Cells were collected in a sterile 15 mL tube, washed, and counted using a Cellometer (Nexcelom).

Magnetic cell separation
CX3CR1+ positive cells were isolated using BioLegend’s Magnetic Cell Separation System, MojoSort™ (Cat. No. 480055). Granulocytic cells were depleted using magnetic nanoparticles directly conjugated to anti-Ly-6G antibody. The negative fraction was subsequently incubated with biotinylated anti-CX3CR1 antibody, followed by washing and incubation with Streptavidin conjugated nanoparticles.

For more information, please visit: biolegend.com/mojosort

Cell differentiation and toll-like receptor stimulation
After magnetic cell isolation, cells were cultured in 24-well plates for 7 days at a density of 1-2 x 10^6/mL in complete RPMI 1640 media, supplemented with GM-CSF (10 ng/mL) or GM-CSF/IL-4 (Cat. No. 576302, 10 ng/mL; Cat. No. 574302, 15 ng/mL). Media was replenished every 2 days. After 7 days in culture, cells were stimulated with LPS (50 µg/mL) or CpG ODN 2395 (5 µM) for 12 to 16 hours. After stimulation, the supernatant was collected and kept at -20°C until processing for cytokine and chemokine detection. Cells were recovered from the plate and analyzed by flow cytometry.

Flow cytometry analysis
Cells were processed and stained following BioLegend’s protocols. Briefly, single cell suspensions were prepared from bone marrow as described previously, or lymphoid organs, and red cells were lysed using Red Blood Cell Lysis Buffer (BioLegend Cat. No. 420301, diluted 1 in 10). Cells were washed, counted, and resuspended at the appropriate concentration for staining. Non-specific binding was blocked using TruStain FcX™ (anti-mouse CD16/163) antibody (Cat. No. 101319). We now recommend TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (Cat. No. 156603, clone S17011E) for higher blocking capacity. Following Fc blocking, cells were stained with anti-CD115 (Cat. No. 135505), CX3CR1 (Cat. No. 149007), CD80 (Cat. No. 107627), Ly-6G (Cat. No. 127625), Ly-6C (Cat. No. 128023), and 7-AAD (Cat. No. 420403). Cells were acquired with an LSR Fortessa™ (BD Biosciences) and analyzed using FlowJo software.

Cytokine and chemokine measurement
Cytokine and chemokine profile was characterized using BioLegend’s LEGENDplex™ system. A detailed protocol has been published. Briefly, LEGENDplex™ is a bead-based immunoassay that employs the same basic principle as sandwich immunoassays. Capture beads, which can be differentiated by size and internal fluorescence intensity, are conjugated to antibodies specific to a particular analyte. A panel of defined capture beads is incubated with the sample containing target analytes specific to the capture antibodies. Then a biotinylated detection antibody cocktail is added, which leads to the formation of capture bead-analyte-detection antibody sandwiches. Finally, streptavidin-phycoerythrin (PE) is added, which binds to biotinylated detection antibodies, providing fluorescent signal intensities in proportion to the amount of bound analyte. The PE fluorescent signal of analyte-specific bead regions is quantified using flow cytometry, and the concentrations of particular analytes are determined using data analysis software and the standard curve generated in the assay.

For more information, please visit: biolegend.com/legendplex

In vivo cell transfer
CX3CR1+ cells were isolated as described above, and labeled with CFSE following BioLegend's recommendations. Recipient mice were anesthetized with isoflurane and 10^6 cells were injected retro-orbitally in 100 µL of PBS. Control animals received 100 µL of PBS only. 72 hours post-transfer, the mice were sacrificed and spleen, bone marrow, and lymphoid organs were collected and processed for flow cytometry analysis. Cells were stained with anti-CD115 (Cat. No. 135505), CX3CR1 (Cat. No. 149007), I-A/I-E (MHC II) (Cat. No. 107627), Ly-6G (Cat. No. 127625), Ly-6C (Cat. No. 128023), and 7-AAD (Cat. No. 420403). Cells were acquired with an LSR Fortessa™ (BD Biosciences) and analyzed using FlowJo software.

All reagents are from BioLegend, unless specified otherwise.

For more information, please visit: biolegend.com/technical_protocols
Results

Effective enrichment of bone marrow CX3CR1⁺ cells using MojoSort™ positive selection

The bone marrow is found within the central cavities of axial and long bones. It consists of hematopoietic tissue islands and adipose cells surrounded by vascular networks embedded in trabecular bone. It is the major hematopoietic organ as well as a primary lymphoid tissue, responsible for the production of erythrocytes, granulocytes, monocytes, lymphocytes and platelets⁶. Despite its importance in blood and other disorders, its complexity has made it difficult to fully characterize all the cell precursors and their physiological functions.

Our aim with this study was to examine a relatively unknown precursor, characterized by expression of CX3CR1, and its capacity to generate cells that could display either macrophage or dendritic cell characteristics, depending on the culturing conditions induced by GM-CSF alone, or GM-CSF and IL-4. However, initial experiments soon revealed the challenges of isolating such population from bone marrow, as there were large proportions of unrelated cells that could potentially reduce the number of CX3CR1⁺ cells to be selected by the magnetic isolation. A particularly large proportion of these unwanted cells were identified as granulocytic cells, defined by high expression of Ly-6G (Figure 1).

Figure 1. The percentage of Ly-6G⁺ cells in mouse bone marrow can be relatively large; it may be advantageous to deplete this population before enriching CX3CR1⁺ cells.
Thus, we designed an isolation strategy to first deplete these granulocytic cells, using Ly-6G conjugated magnetic nanoparticles. This would maximize the chance to obtain higher purity, while maintaining an acceptable yield when trying to perform positive selection of CX3CR1+ cells. The positive selection would be performed using CX3CR1 biotinylated antibodies, followed by magnetic Streptavidin nanoparticles. The complete experimental design, including the subsequent cell culturing in 24-well plates in the presence of the different cytokine supplements is shown in figure 2.

Following this strategy, we were able to enrich the CX3CR1 positive fraction more than 10 fold. Working with low frequency cells is always a challenge, as the number of positive events is scarce and cell loss is difficult to overcome. However, we were able to optimize the protocols and reagents used, and found a robust strategy that worked consistently even for such a rare cell population that does not surpass 7% of total cells in murine bone marrow (Figure 3).

Figure 2. Experimental layout to isolate CX3CR1+ cells, differentiate them to dendritic cells or macrophages, and adoptively transfer them to a recipient mouse and monitor cell migration.

Figure 3. MojoSort™ enrichment process multiplies the percentage of CX3CR1+ cells over 10-fold after positive selection.
Surface phenotype of differentiated cells after TLR stimulation

After successfully enriching CX3CR1+ cells, we differentiated them using media supplemented with either GM-CSF alone or GM-CSF/IL-4. Although the field of bone marrow-derived cells is under constant research, there is an overall understanding that the addition of these recombinant proteins in the media is expected to differentiate the cells towards macrophages (GM-CSF) or dendritic cells (GM-CSF/IL-4). Given that there is still a need to fully characterize bone marrow-derived, terminally differentiated cells, and considering the differences reported between these two cell types, we sought to analyze their surface phenotype in the absence or presence of TLR stimulation, as well as their cytokine and chemokine profile in vitro.

Figure 4 shows expression of CX3CR1 and CD115 in GM-CSF cultured cells, and CX3CR1 and CD11c in GM-CSF/IL-4 cultured cells (left and right top panels, respectively). We observed a distinct phenotype for these two cell cultures, which supports the idea that they differentiate towards different cell types. Furthermore, when the cells were stimulated with TLR agonists, such as LPS (TLR4 agonist) or CpG (TLR9 agonist), they responded by upregulating MHC II, CD80, and CD86. This is expected in antigen presenting cells (APCs) as they express several TLRs and undergo further maturation when stimulated with TLR agonists. A classical landmark of APC maturation is the upregulation of MHC II and co-stimulatory molecules (CD80, CD86) upon TLR stimulation. We also observed a difference in the expression pattern when comparing the two cultures, with unstimulated GM-CSF derived cells (grey histograms) expressing lower levels of MHC II and CD80, but higher levels of CD115. However, LPS (red histograms) and CpG (blue histograms) stimulated cells are capable of upregulating MHC II and costimulatory molecules to the same extent. Interestingly, GM-CSF-derived cells downregulate CD115, to the same low levels observed in GM-CSF/IL-4-derived cells, which further supports the evidence that the cells derived from the different culture conditions develop into different cell types.

Figure 4. GM-CSF and GM-CSF/IL-4 derived cells from CX3CR1+ precursors show distinct surface phenotypes (dot plots). Both cultures are able to upregulate MHC II, CD80, and CD86 after TLR ligand stimulation.
Cytokine and chemokine profiles following TLR stimulation

Following the culturing methods described above, we examined the cytokine profile for these cells. Using our LEGENDplex™ multiplexing bead-based system, we analyzed both inflammatory cytokine and chemokine production from the macrophage and dendritic cell populations following stimulation with LPS or CpG. As shown in figure 5, macrophages express a distinct cytokine profile compared to dendritic cells. While macrophages upregulated expression of IL-1α, IL-1β, IL-12p40, IL-12p70, IL-27, IFN-β, IP-10, KC, MIP-1α, and MIP-1β, dendritic cells produced increased amounts of IL-23, BLC, and MIP-3α. In addition, LPS elicited a stronger cytokine and chemokine profile compared to CpG stimulation.

Figure 5. LEGENDplex™ cytokine and chemokine profile of mouse bone marrow-derived macrophages and dendritic cells. Numbers reflect fold-increase based on pg/mL values of stimualted cells over non-stimulated cells.

MojoSort™ isolated cells retain functional ability to migrate

We also assessed the function of these bone marrow CX3CR1+ precursors in vivo by labelling them with CFSE and injecting them into recipient mice. Control mice were treated with PBS. After 3 days, spleen, intestine (data not shown) and bone marrow were harvested and stained. As seen in Figure 6, CX3CR1+ CFSE-labeled cells from the donor mice successfully migrated to the bone marrow, demonstrating that their migratory capacity was unaffected by the MojoSort™ protocol.

Figure 6. Positively isolated CX3CR1 cells were labelled with CFSE and transferred into C57BL/6 mice via retro-orbital injection. On day 3, the recipient mouse bone marrow was isolated and stained for CD11b, F4/80, and CX3CR1.
The function of macrophages and dendritic cells is critical for a number of disease outcomes, as well as normal, physiological conditions making it vital to properly characterize them. Helft et al. have previously looked at cell culturing methods and the heterogeneity of bone marrow-derived macrophages and dendritic cells. Realizing that these cells share overlapping markers, it benefits researchers to look at more phenotypic characteristics, such as comprehensive cytokine and chemokine expression profile, upon pattern recognition receptors stimulation, and other critical stimuli. This is particularly true of bone marrow CX3CR1+ precursors, as less is understood about them compared to their peripheral counterparts. Following isolation of these cells with MojoSort, we differentiated them with GM-CSF (macrophages) or GM-CSF/IL-4 (dendritic cells). We analyzed surface expression of markers like CD80, CD86, and MHC II, with each cell demonstrating upregulation of costimulatory molecules following stimulation with LPS or CpG. We also examined cytokine production using LEGENDplex. Each population exhibited distinct cytokine profiles depending on the type of TLR agonist used. Finally, we demonstrated that CX3CR1+ cells are capable of migration to the bone marrow after adoptive transfer in vivo. The derivation of large numbers of macrophages and dendritic cells from bone marrow has become a staple of researchers across several fields of immunology. Our experiments help to characterize these populations to provide a better understanding of their phenotype, function, and abilities.

References