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Lysophosphatidic acid converts monocytes into macrophages in both mice and humans

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Key Points

- LPA converts monocytes into macrophages.
- LPA mediates macrophage formation via Akt/mTor pathway and PPARγ is master regulator of LPA derived macrophages.

Abstract

Monocytes and macrophages represent critical arms of the innate immune system and are considered regulators and effectors of inflammation and innate immune response. Monocytes can mobilize from bone marrow, traffic to their required destination and differentiate into effector cells depending on the local tissue environment to perform multiple roles during infection or inflammation, making them an important component of body's immune defense. Macrophages have diverse roles in tissue homeostasis, development as well as tissue repair following injury. Adult bone-marrow monocytes can give rise to tissue resident macrophages during infection or inflammatory reactions, besides self-replication of tissue resident macrophages. Lysophosphatidic acid (LPA), a lipid by-product of autotaxin activity is involved in cancer, vascular defects and neural tissue but largely unexplored in immune system. Here, we reveal an unexpected function of LPA that transfigures $CD11b^+$ murine monocytes into $F4/80^+$ macrophages. LPA stimulated Akt/m-TOR signaling is critical for LPA mediated macrophage development in mice. Additionally, transcriptome analysis reveals that PPARγ is the key transcriptional regulator in the development of LPA induced macrophages. In humans, LPA mediates macrophage formation following similar pathways. These findings identify critical role for LPA in regulating innate immune system.

Keywords: monocytes, macrophages, Akt, mTor and LPA

Introduction

Monocytes and macrophages constitute important components of mononuclear phagocytic system (MPS) and play diverse roles during infection, inflammation, tissue injury and repair. Monocytes differentiate into macrophages following stimulation with cytokines and/or microbial molecule [1,2]. Macrophages play central role in the maintenance of tissue homeostasis, development and its restoration after injury, as well as the initiation and resolution of innate and adaptive immunity. Though macrophages were long considered to be derived from differentiation of bone marrow monocytes, recent studies have proved that tissue resident macrophages are derived from yolk sac macrophages[3], fetal liver macrophages[4], can self-replicate from local proliferation[5] and do not solely depend on adult bone-marrow monocytes (BM-MOs). However, during homeostatic adaptations, injury and inflammation macrophages of different phenotypes can be recruited from the monocyte reservoirs of blood, spleen and bone marrow[6,7]. Lysophosphatidic acid (LPA), a bioactive phospholipid exerts numerous cell responses varying from cell motility, neuropathic pain, infertility, cardiovascular disease, inflammation, fibrosis, and cancer [8-10]. This diversity is mediated by broad and overlapping expression patterns and multiple downstream signaling pathways activated by identical LPA receptors but this lipid molecule remains little known in immune system[9,11-14]. Here we reveal, that Lysophosphatidic acid (LPA), a small lipid molecule converts monocytes into macrophages in both mouse and human.

Study design

Monocytes were isolated from bone marrow of C57BL/6 mice. Monocytes were isolated using negative selection of mouse monocytes (CD11b positive) using magnetic beads from Stem Cell Technologies. Isolated bone marrow monocytes were incubated in presence of LPA, M-CSF, and Akt, mTor or PPARy inhibitors for the indicated time periods and

harvested for flow cytometry, gene expression, or immunoblotting analyses (for detailed methods see Supplemental Methods).

Results and discussions

To explore the role of LPA on monocytes we added LPA on CD11b positive monocytes isolated from bone marrow of 6-8 weeks old C57BL/6 mice and observed after 5 days, to our surprise we found that those monocytes were surviving and phenotypically been converted into a different cell type. Geimsa staining revealed their morphology to be similar to macrophages (Figure 1A-B). F4/80 is one of the most specific cell-surface markers for murine macrophages[15-18]. Our immunoblot, immunofluorescence and FACS analysis showed F4/80 expression in these macrophages (Figure 1B-D; Supplemental figure 1E) confirming these cells were macrophages. Further qPCR analysis showed increased expression of F4/80 and CD11b surface markers in a dose dependent manner in LPA treated monocytes, M-CSF, a well-known macrophage inducing growth factor[19-21] was used as positive control (Figure 1E-F; Supplemental figure 1A-B) furthermore LPA treated monocytes did not show much enhancement in proliferation of differentiating F4/80 macrophages (Supplemental figure 2D-E). To further check the effect of LPA on monocytes other than bone marrow origin, we isolated and treated splenic monocytes with LPA and found LPA induced macrophage formation in splenic origin monocytes as well (Supplemental figure 1C-D).

Immunoblot analysis for phosphorylated proteins showed increased phosphorylation of Akt in monocytes after LPA addition with further downstream mTor phosphorylations (Figure 1G-H; Supplemental Figure 2A). To confirm the involvement of Akt/mTor pathway in LPA mediated macrophage formation, we pretreated mouse monocytes with specific Akt inhibitor (LY294002) and next added LPA on these monocytes to convert them into macrophages. Morphological observation indicated reduced formation of macrophages on Akt inhibitor pretreatment (Figure 1I; Supplemental Figure 2B-C). FACS analysis showed drastic

reduction in percentage of F4/80 expressing cells in LPA treated monocytes while it did not affect macrophage formation from M-CSF or LPS at the similar dose (Figure 1J-K). Mouse monocytes pretreated with mTor inhibitor (Rapamycin) followed by LPA, M-CSF or LPS addition showed no significant effect on M-CSF mediated macrophage formation while it inhibited macrophage formation in LPA treated monocytes at similar dose (Figure 1J; Supplemental Figure 2C).

Thioglycollate injection in the pleural and peritoneum cavity elicits higher number of monocytes from circulation [5,22,23]. C57BL/6 mice were injected with 3% thioglycollate in peritoneum and pleural cavity followed by LPA injection in cavities, the total cell population isolated from the peritoneal and pleural cavity 3 days after the thioglycollate injection showed higher percentage of F4/80 expressing cells in LPA injected mice compared to PBS injected mice both in peritoneum and pleural cavity (Figure 2A) confirming that LPA converts monocytes into macrophages *in vivo*.

Lysophosphatidic acid (LPA) is synthesized from lysophosphatidylcholine (LPC) by the catalytic activity of Autotaxin (Atx) in biological systems [9,24]. Next to validate our finding with conditional Atx transgenic mice which produces more LPA due to higher expression of Atx in circulation [25] we injected 3% thioglycollate in the peritoneal and pleural cavities of wild type (WT) and ATX transgenic (ATX-Tg) mice. Our analysis showed higher percentage of F4/80 expressing cells in ATX-Tg mice compared to WT (Figure 2B-C).

RNA sequencing of isolated monocytes and LPA converted macrophages was performed and in this regard, Supplemental Figure 4A highlights a set of genes whose expression distinguished between monocytes and LPA mediated macrophages, spotlighting six biological categories (Figure 2D; Supplemental Figure 4B-E) with the predicted functionality of each subset. F4/80 (EMR1), was highly expressed in LPA converted macrophages along

with PPARγ transcription factor as revealed with network analysis suggesting PPARγ as a ‘‘master’’ TF for LPA mediated macrophages (Supplemental Figure 4F).

qPCR confirmed higher expression of PPARγ along with macrophage specific TFs (Figure 2E; Supplemental Figure 5A-B). Further, ChIP-qPCR analysis revealed measurable recruitment of endogenous PPARγ at the PPARγ binding sites[26] in LPA derived macrophages compared with control monocytes (Supplemental figure 5C). PPARγ inhibition drastically reduced macrophage population (Figure 2F; Supplemental Figure 6A). Interestingly, Akt or mTor inhibitor pretreatment reduced PPARγ expression during LPA mediated monocyte differentiation to macrophages (Figure 2G). To check functional implication of these macrophages we stimulated LPA derived macrophages (LDMa) with LPS, and found elevated levels of cytokines and chemokines secretion similar to M-CSF derived macrophages (Supplemental Figure 7A-F).

To determine whether murine development of macrophages from monocytes were coherent with LPA effect on human monocytes, CD14 positive monocyte isolated from human PBMCs were treated with different doses of LPA. These LPA generated macrophages showed higher expression of CD68 as seen by flow cytometry and CD14, CD64, CD68 and CD206 by qPCR comparable to human M-CSF generated macrophages (Figure 2H-L; Supplemental Figure 6B). Further qPCR showed that 2.5μM LPA was optimum for human macrophage formation with higher expression of CD68, a macrophage marker and CD14 (Supplemental figure 9A-B). Further to confirm Akt/mTor pathway found in LPA mediated mouse macrophages formation in human macrophage formation from CD14 monocytes, we pretreated CD14 monocytes with Akt inhibitor followed by addition of LPA. We found that Akt inhibitor treated CD14 monocytes severely reduced the percentage of macrophages on further LPA treatments (Figure 4L). qPCR analysis also confirmed enhanced expression of

PPARγ mRNA in human monocytes. Monocytes pretreated with PPARγ inhibitor did not form macrophages from CD14 monocytes in presence of LPA confirming it role in human macrophages formation as well (Figure 2M-N; Supplemental figure 8A-B) establishing PPARγ as the key master regulator in LPA mediated macrophage development.

This work demonstrates unique potential of LPA to produce macrophages *ex vivo* and *in vivo* and in both mice and humans. Macrophages are an important component of innate immune system and produce a diverse range of biologically active molecules participating in both beneficial and detrimental outcomes in inflammation, thereby making it an important avenue for therapeutic interventions targeting macrophages and their products may open ways for controlling inflammatory diseases[16,22]. Akt-mTor or PPARγ is critical in LPA mediated macrophage formation and differentiation as seen *in vitro*, *in vivo*. Furthermore, transcriptome analysis characterizes these new LPA mediated macrophages.

This finding may ultimately provide a foundation for LPA in innate system. Interestingly, in many types of cancer and tumors LPA is highly upregulated[9,14,27], this work facilitates future possible role of LPA in tumor associated macrophages. Furthermore, a better understanding is needed of the underlying mechanisms for this reprogramming.

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Author contributions

R.R. contributed to project planning, experimental work, data analysis, and writing the manuscript. V.R. contributed to project planning, data analysis, writing the manuscript and overall supervising this study.

Conflict of interest

The authors declare no conflict of interest.

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Figure Legends:

Figure 1. LPA converts murine monocytes into macrophages via Akt-mTor pathway. **(A)** Representative phase contrast images of mouse monocytes cultured in RPMI only (control) or in RPMI with LPA (10µM) or M-CSF (10ng/ml) upto 7 days. **(B)** Left panel, Geimsa stained images of mouse monocytes cultured *in vitro* in presence of LPA or M-CSF for 7 days. Figures are representative of 10 images (n=10). Right panel, Immunoblot showing levels of F4/80 and b-actin in control (RPMI), LPA and M-CSF treated monocytes. **(C)** FACS plots showing proportion of CD11b and F4/80 positive macrophages in monocytes treated with LPA or M-CSF for 5 days. **(D)** Quantification of percentage of CD11b and F4/80 positive cells in monocytes cultured in medium and cells cultured in medium with LPA or M-CSF. **(E, F)** Quantification of CD11b and F4/80 relative mRNA in monocytes (control) and in monocytes cultured for 5 days in medium with different doses of LPA (50 and 10uM) or M-CSF. **(G, H)** Immunoblot showing levels of p-Akt, p-mTor, Akt and mTor in macrophages differentiated from mouse monocytes cultured in medium with LPA at indicated time points. **(I)** Representative geimsa stained images of LPA derived macrophages or M-CSF derived macrophages without or with pretreatment of Akt inhibitor. **(J)** FACS plots showing proportion of CD11b and F4/80 positive macrophages in monocytes cultured in presence of LPA, M-CSF or LPS and monocytes pretreated with Akt inhibitor (LY-294002) and m-Tor inhibitor (Rapamycin) and cultured in medium containing LPA, M-CSF or LPS respectively. **(K)** Quantification of CD11b and F4/80 positive macrophages cultured in medium with only LPA and LPA after pretreatment with Akt or m-Tor inhibitor respectively. Scale bars, 100

μm. Graph presents mean ± SD of five experiments per condition. Error bars represent SD. Student's t test was used for all statistical analyses (***P<0.001, **P < 0.01, *P < 0.05).

Figure 2. LPA generates macrophages *in vivo* and in humans through a common transcription factor PPARγ both in mice and human (A) FACS plots showing percentage of CD11b and F4/80 positive macrophages elicited in peritoneal and pleural cavities of C57BL/6 mice injected with 3% thioglycollate in absence or presence of 20μg LPA. **(B)** FACS plots showing percentage of CD11b and F4/80 positive macrophages elicited in peritoneal and pleural cavities of FVB wild type (FVB-WT) and FVB-ATX transgenic (FVB-ATX-Tg) mice injected with 3% thioglycollate. **(C)** Proportion of peritoneal macrophages positive for F4/80 CD11b in FVB WT and FVB ATX-Tg mice. **(D)** Heat map showing the expression of surface markers, transcription factors and cytokines related genes in monocytes and LPA derived macrophages (LDMa). **(E)** Quantification of PPARγ mRNA in mouse monocytes and macrophages derived from LPA or M-CSF treated mouse monocytes for different days. **(F)** FACS plots showing percentage of CD11b and F4/80 macrophages in monocytes pretreated with different doses of PPARγ inhibitor and cultured in presence of LPA or M-CSF for 5 days. **(G)** Quantification of PPAR γ in monocytes (control), monocytes treated with LPA and monocytes pretreated with Akt inhibitor or mTor inhibitor and cultured further in medium supplemented with LPA for 5 days. **(H)** Representative phase contrast and geimsa stained images of human monocytes cultured in RPMI only (control) and human monocytes cultured in medium with 2.5 μM LPA and 10ng/ml M-CSF for 7 days. **(I-K)** Quantification of CD14, CD68 and CD206 mRNA in human monocytes and macrophages derived from human monocytes cultured with different doses of LPA or M-CSF at day 5. **(L)** FACS plots showing percentage of CD14 and CD68 positive macrophages in monocytes treated with M-CSF, LPA or Akt inhibitor (LY294002) pretreated monocytes cultured in

medium with LPA for 5 days. **(M)** Quantification of PPARγ relative mRNA in human monocytes and monocytes cultured in medium with LPA or M-CSF for 7 days. **(N)** FACS plots showing percentage of CD14 and CD68 positive macrophages in human monocytes cultured with or without PPARγ inhibitor in presence of LPA for 5 days. Graphs depict mean ± SD of four or five mice. Data are representative of two independent experiments. Error bars represent SD. Scale bars, 100 μm. Student's t test was used for all statistical analyses (***P<0.001).

Figure. 1

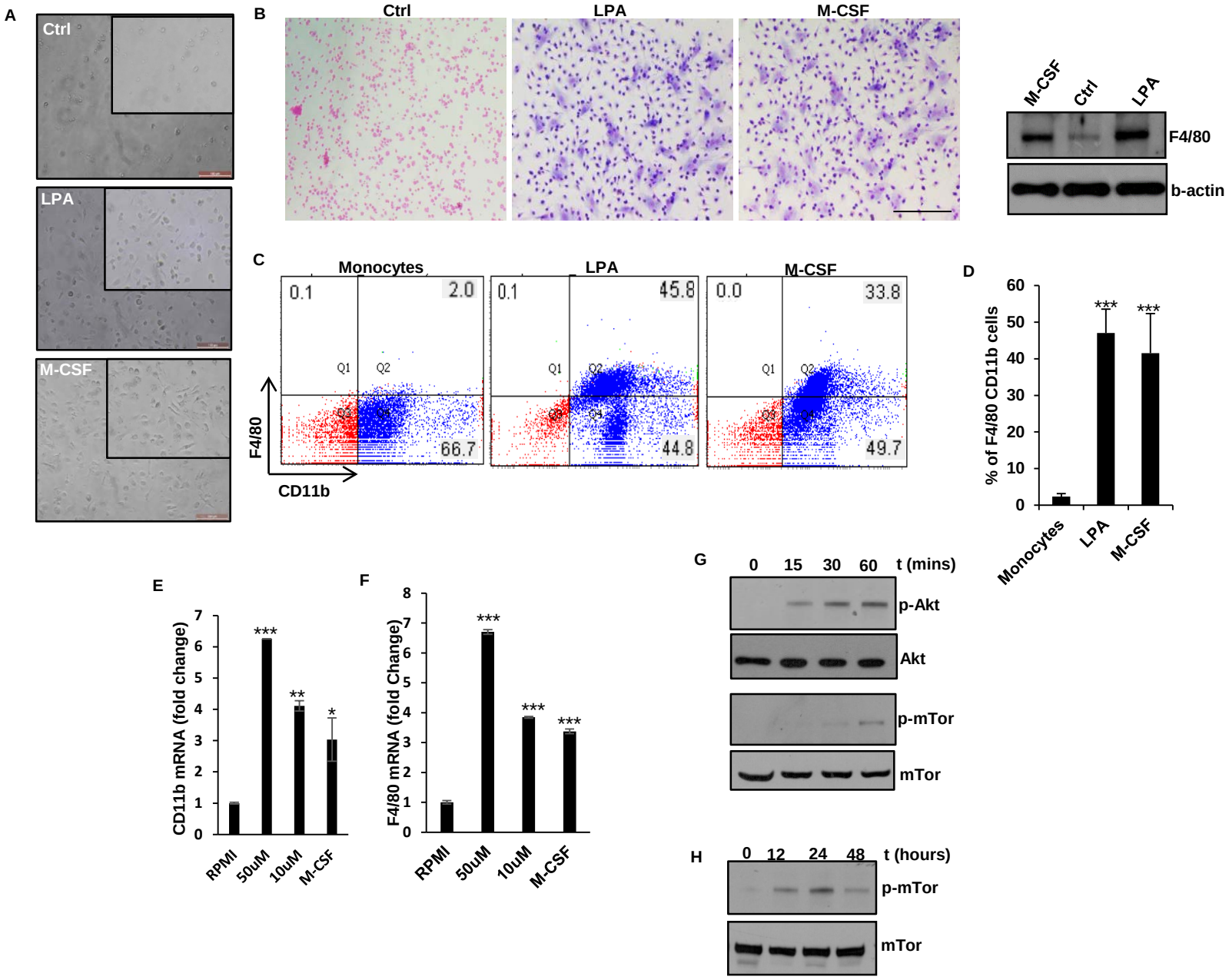
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Figure. 1 Continued….

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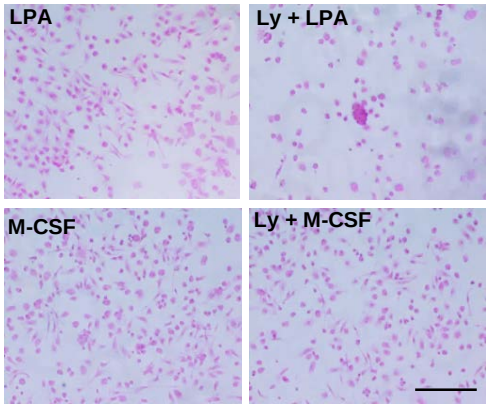

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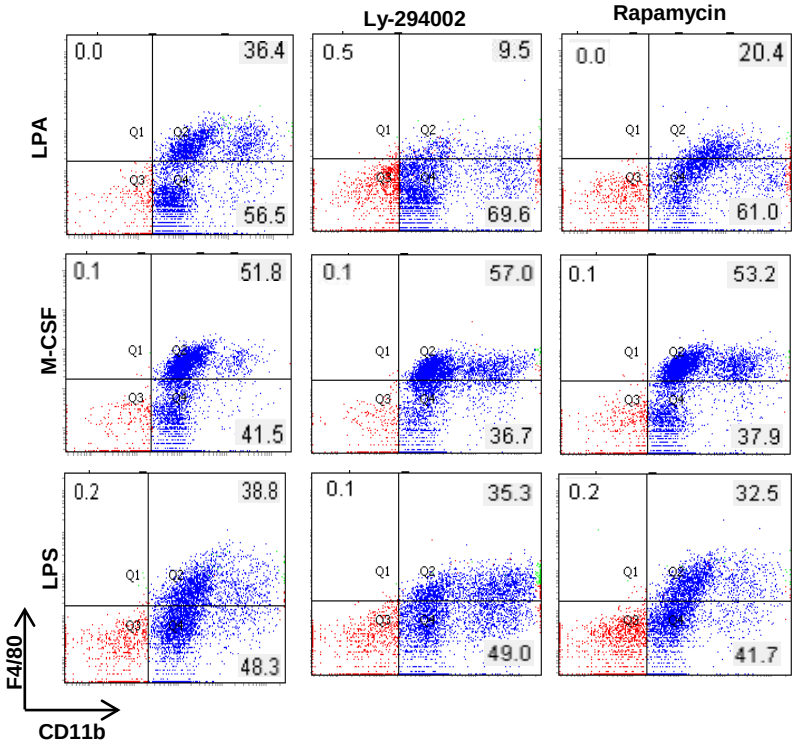

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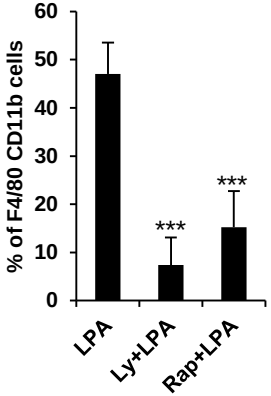
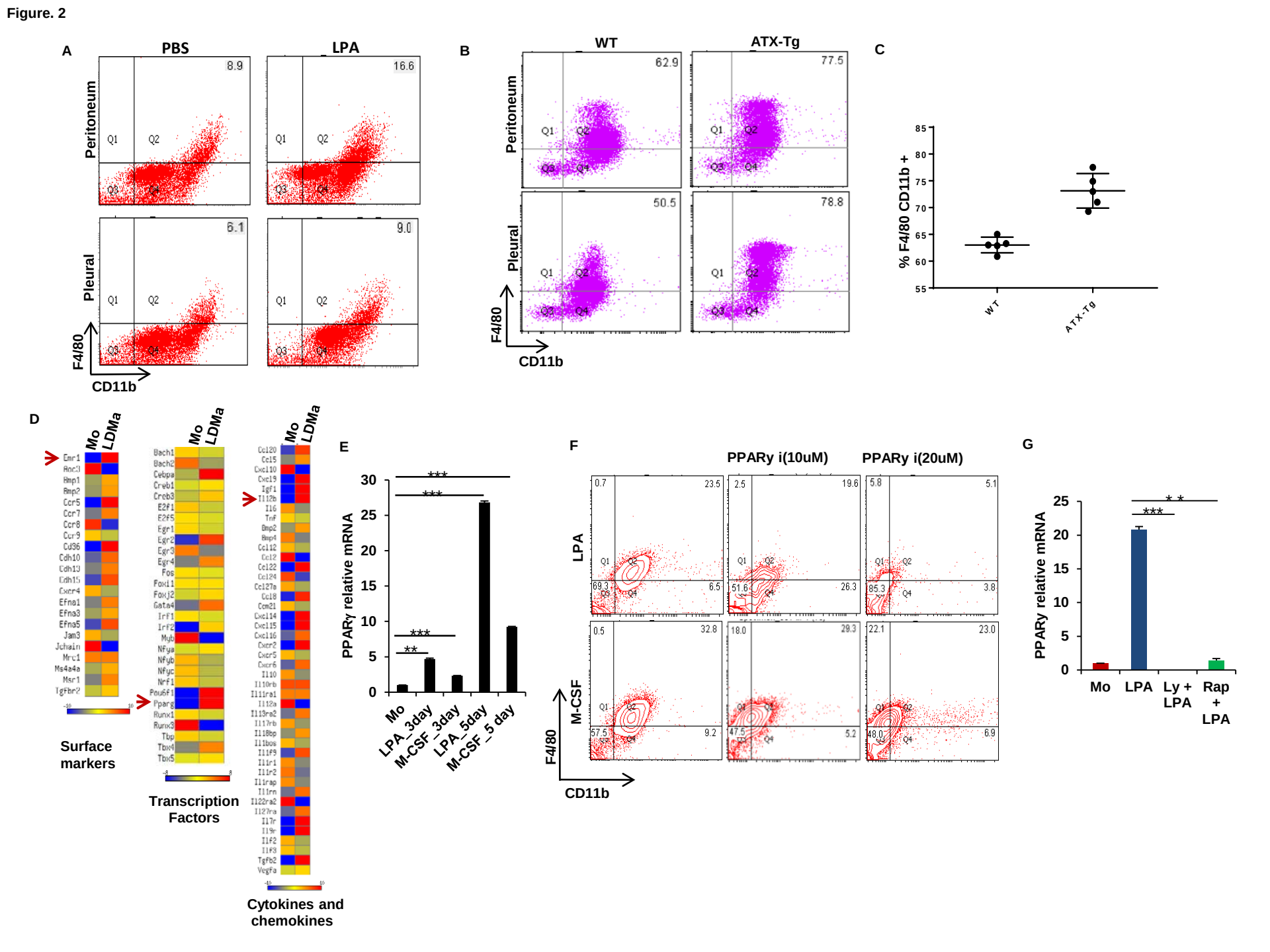


Figure. 2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D
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LDMa
Surface markers
Transcription Factors
Cytokines and chemokines
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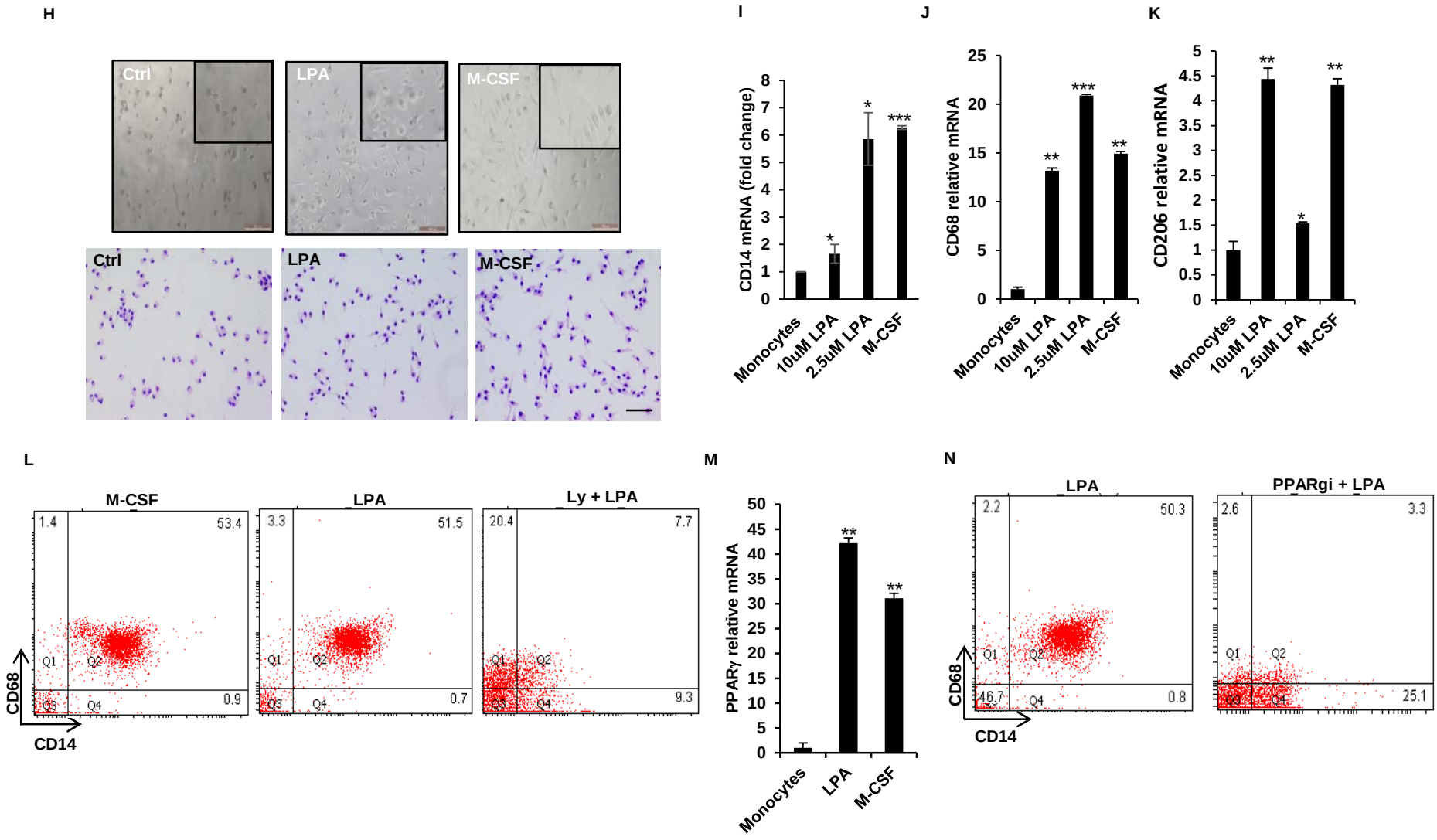

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