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This information is current as of September 16, 2013.

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*J Immunol* 2013; 191:3090-3099; Prepublished online 16 August 2013;  
doi: 10.4049/jimmunol.1300772  
<http://www.jimmunol.org/content/191/6/3090>

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# Dendritic Cell Reprogramming by Endogenously Produced Lactic Acid

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**The demand for controlling T cell responses via dendritic cell (DC) vaccines initiated a quest for reliable and feasible DC modulatory strategies that would facilitate cytotoxicity against tumors or tolerance in autoimmunity. We studied endogenous mechanisms in developing monocyte-derived DCs (MoDCs) that can induce inflammatory or suppressor programs during differentiation, and we identified a powerful autocrine pathway that, in a cell concentration–dependent manner, strongly interferes with inflammatory DC differentiation. MoDCs developing at low cell culture density have superior ability to produce inflammatory cytokines, to induce Th1 polarization, and to migrate toward the lymphoid tissue chemokine CCL19. On the contrary, MoDCs originated from dense cultures produce IL-10 but no inflammatory cytokines upon activation. DCs from high-density cultures maintained more differentiation plasticity and can develop to osteoclasts. The cell concentration–dependent pathway was independent of peroxisome proliferator–activated receptor  $\gamma$  (PPAR $\gamma$ ), a known endogenous regulator of MoDC differentiation. Instead, it acted through lactic acid, which accumulated in dense cultures and induced an early and long-lasting reprogramming of MoDC differentiation. Our results suggest that the lactic acid–mediated inhibitory pathway could be efficiently manipulated in developing MoDCs to influence the immunogenicity of DC vaccines. *The Journal of Immunology*, 2013, 191: 3090–3099.**

**D**endritic cells (DCs) are potent APCs, and due to their ability to induce and orchestrate T cell activation, these cells are important candidates to be used as vaccine adjuvant or to be targeted by immunomodulatory therapies (1–3). Ex vivo–generated monocyte-derived DCs (MoDCs) have been tested as therapeutic tumor vaccines in various clinical trials, and several results indicated safety and immunogenicity of these vaccines and, in some cases, increased survival of patients in late disease stages (1, 3). It is evident, however, that the DC-based tumor vaccines are expected to improve in overall efficacy (1, 4, 5). MoDCs, when injected into patients, showed surprisingly little migratory potential (6, 7). In addition, the T cell stimulatory potential of DC vaccines should be improved to counteract the immunosuppressive tumor milieu that, partly by downmodulating DC activities, promotes tolerance (8–11).

MoDCs are usually considered as a homogeneous cell population in the expression of cell surface markers and in function. We have, in contrast, previously reported coexisting, functionally different subsets in MoDC cultures generated with GM-CSF and IL-4: CD1a<sup>+</sup>CD14<sup>−</sup>CD209<sup>+</sup> DCs that produced IL-12 upon activation and phagocytic non-IL-12–producing CD1a<sup>−</sup>CD14<sup>+</sup>CD209<sup>+</sup> DCs (12). Importantly, we observed significant individual variability of CD1a<sup>+</sup>/CD1a<sup>−</sup> MoDC ratios among healthy donors (12). MoDC variability might affect the efficiency of DC-based tumor vaccines, which can be severely compromised in cases where predominantly the noninflammatory CD1a<sup>−</sup> DC type develops. Nevertheless, other research groups have not confirmed our observation on coexisting MoDC subsets in GM-CSF and IL-4–supplemented cultures. When DCs were generated from CD34<sup>+</sup> hematopoietic stem cells using GM-CSF, Flt3 ligand, and TNF, functionally diverse CD1a<sup>+</sup>CD14<sup>−</sup> and CD1a<sup>−</sup>CD14<sup>+</sup> DCs developed (13); however, studies that analyzed CD1a expression in the presence of GM-CSF and IL-4 reported a homogeneous CD1a<sup>+</sup>CD14<sup>−</sup> MoDC differentiation (14, 15). Such variability between MoDCs originated from different blood donors in our laboratory, and MoDCs generated in different laboratories suggested the possibility that differences in the experimental procedure might have a strong influence on MoDC development.

In this paper, we describe a novel autocrine mechanism that induces a potent anti-inflammatory program during MoDC differentiation in a cell culture density–dependent manner and decreases the commitment toward DC development. Higher density led to the appearance of CD1a<sup>−</sup> DCs, the production of the anti-inflammatory cytokine IL-10 but little or no IL-12 upon TLR stimulation, and the preserved ability of the cells to differentiate into osteoclasts. Decreasing cell culture density eliminated the IL-10 production and primed the cells to produce very high levels of IL-12, IL-23, and TNF upon activation, to induce Th1 polarization, and to migrate toward the lymphoid tissue chemokine CCL19. The mediator molecule responsible for the density-dependent differentiation

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Received for publication March 21, 2013. Accepted for publication July 18, 2013.

This work was supported by the Swedish Medical Research Council and the Clas Groschinsky Minnesfond. A.N. was supported by the Bodossakis Foundation. T.F. was supported by the Social Renewal Operational Programme Grant TAMOP 4.2.2/B-10/1-2010-0024 and a fellowship from the European Federation of Immunological Societies and Immunology Letters.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; 15-LOX, 15-lipoxygenase; MoDC, monocyte-derived dendritic cell; PPAR $\gamma$ , peroxisome proliferator–activated receptor  $\gamma$ ; TRAP, tartrate-resistant acid phosphatase.

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switch proved to be lactate that accumulated in dense cultures. Our results thus demonstrated a key role for glucose metabolic pathways in the orientation of the developing MoDCs toward inflammatory, Th1 stimulatory, or toward noninflammatory activities. When MoDCs are generated for tumor therapies, this inhibitory pathway might be efficiently manipulated to increase the immunogenicity of DC vaccines.

## Materials and Methods

### MoDC cultures

Blood samples were obtained from healthy blood donors (buffy coats). The ethical committee at the Karolinska Institutet approved our studies involving human samples. Monocytes were isolated using Ficoll centrifugation (Lymphoprep; Axis Shield, Oslo, Norway) and magnetic separation with anti-CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were cultured using a range of cell concentrations between  $2 \times 10^6$  and  $0.125 \times 10^6$  cells/ml in RPMI 1640 medium (not AIM-V, not very low endotoxin-rated, endotoxin level  $\leq 0.04$  endotoxin unit/ml) supplemented with 10% FCS (endotoxin level  $\leq 5$  endotoxin unit/ml), both from Life Technologies, 75 ng/ml (675 IU/ml) GM-CSF (Gentaur, Kampenhout, Belgium), and 50 ng/ml (250 IU/ml) IL-4 (PeprroTech, London, U.K.) or as stated in the text. At day 3, 50% of the medium was replaced, and new cytokines were added and the cells were cultured until day 6. For DC activation LPS (250 ng/ml), CL075 (1  $\mu$ g/ml), both from InvivoGen (San Diego, CA), or recombinant CD40L (5  $\mu$ g/ml; PeprroTech) were used. GW-9662 and baicalein (both from Calbiochem, Merck, Darmstadt, Germany) were used as a concentration of 0.1 and 0.4  $\mu$ g/ml, respectively. Oxamic acid (Sigma-Aldrich, St. Louis, MO) was used in the concentration of 1 mg/ml (pH adjusted to 7.4), or alternatively, the drug was applied as a 1-h pretreatment before the initiation of the MoDC culture in the concentration of 2 mg/ml. When oxamic acid was applied continuously, we detected a 10.8+3.5 increase in the percentage of apoptotic cells by day 3 as compared with untreated cultures ( $n = 4$ ) and a 19+6% decrease in the total number a viable MoDCs. When oxamic acid was applied as a 1-h pretreatment, we did not observe an increase in apoptotic cell number or a decrease in total MoDC numbers by day 3 as compared with untreated cultures ( $n = 4$ ). Glucose-free RPMI 1640 medium was purchased from Life Technologies; D-glucose, D-galactose, and L-lactic acid were purchased from Sigma-Aldrich.

### Osteoclast cultures

MoDCs generated using different culture densities were collected at day 6 and recultured at  $0.5 \times 10^6$  cells/ml in DMEM supplemented with 10% FCS, 25 ng/ml M-CSF, and 50 ng/ml RANKL (R&D Systems, Minneapolis, MN). Tartrate-resistant acid phosphatase (TRAP) activity was analyzed at day 14, using the leukocyte acid phosphatase kit (Sigma-Aldrich). TRAP-positive cells with three or more nuclei were counted as osteoclasts. Bone resorption activity was tested on 24-well osteoassay plate coated with a synthetic calcium phosphate surface that mimics bone tissue (Corning, Tewksbury, MA) using culture conditions as described above. Resorption areas were analyzed using NIS-Elements software from Nikon in at least four randomly selected fields per sample.

### Flow cytometry

FITC-labeled anti-CD14 (clone M5E2), anti-HLA DQ (clone Tu169), and anti-CD86 (clone 2331 FUN-1), PE-labeled anti-CD1a (clone HI149) and anti-CXCR4 (clone 12G5), allophycocyanin-labeled anti-CD209 (clone DCN46), and PECy5-conjugated anti-CD83 (clone HB15e) Abs were purchased from BD Pharmingen (San Diego, CA), whereas FITC-conjugated anti-CCR7 (clone 150503) and APC-conjugated anti-CCR5 (clone CTC5) were from R&D Systems. T cell cytokines were analyzed using allophycocyanin-labeled anti-IFN- $\gamma$  (clone 45-15) and anti-IL-10 (clone JES3-9D7), FITC-labeled anti-IL-17 (clone CZ8-23G1) and PE-labeled anti-IL-4 (clone 7A3-3), all from Miltenyi Biotec, and the Cytotfix/Cytoperm kit (BD Pharmingen) following a 4-h stimulation using PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of BD GolgiPlug used according to the manufacturer's recommendations. Apoptosis was measured using FITC-labeled Annexin-V and propidium iodide (both from BD Pharmingen), and we used CountBright beads (Life technologies) for cell counting. Samples were analyzed using FACSort (BD Biosciences, San Jose, CA) and the FlowJo software 9.2 (Tree Star, Ashland, OR).

### Measurement of cytokines and lactic acid in supernatants

The IL-12 p70, TNF, and IL-10 OptEIA kits were purchased from BD Pharmingen, and IL-23 ELISA was from eBioscience (San Diego, CA). A

glycolysis cell-based assay kit for lactic acid measurements was purchased from Cayman Chemical (Ann Arbor, MI).

### Chemotaxis experiments

MoDC migration was analyzed in HTS-Transwell 96 plates with 5- $\mu$ m pore size (Corning). Cells were LPS-activated in the upper chambers or were left without activation and then migrated toward 50 ng/ml CCL19 or without chemotactic signal for 8 h. Cells that migrated through the membranes were counted using flow cytometry.

### Gene expression analysis

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH), and cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Carlsbad, CA). Gene expression assays were purchased from Applied Biosystems, except the 36B4 housekeeping gene assay (Integrated DNA Technologies, Coralville, IA). PCR was performed using the Applied Biosystem's StepOne Real-Time PCR. Gene expressions were normalized using the 36B4 expression data according to the  $2^{-(Ct \text{ 36B4-Ct gene of interest})}$  formula.

### Lipid measurement

Lipid mediator analyses were performed as described previously (16). Analytical standards and isotopically labeled internal standards (Supplemental Table I) were purchased from Cayman Chemicals, Larodan Fine Chemicals (Malmo, Sweden) or Enzo Life Sciences (Farmingdale, NY). Lipids were extracted from  $0.5 \times 10^6$  pelleted cells lysed in 1 ml methanol or from 500  $\mu$ l supernatant using off-line solid-phase extraction with Oasis HLB 60 mg cartridges (Waters, Milford, MA). Samples were resuspended in 100  $\mu$ l methanol and analyzed on Acquity UPLC system (Waters) using 2.1  $\times$  50 mm BEH C18 column with 1.7- $\mu$ m particle size. Mass spectral detection was performed using a Xevo TQ triple quadrupole mass spectrometer (Waters).

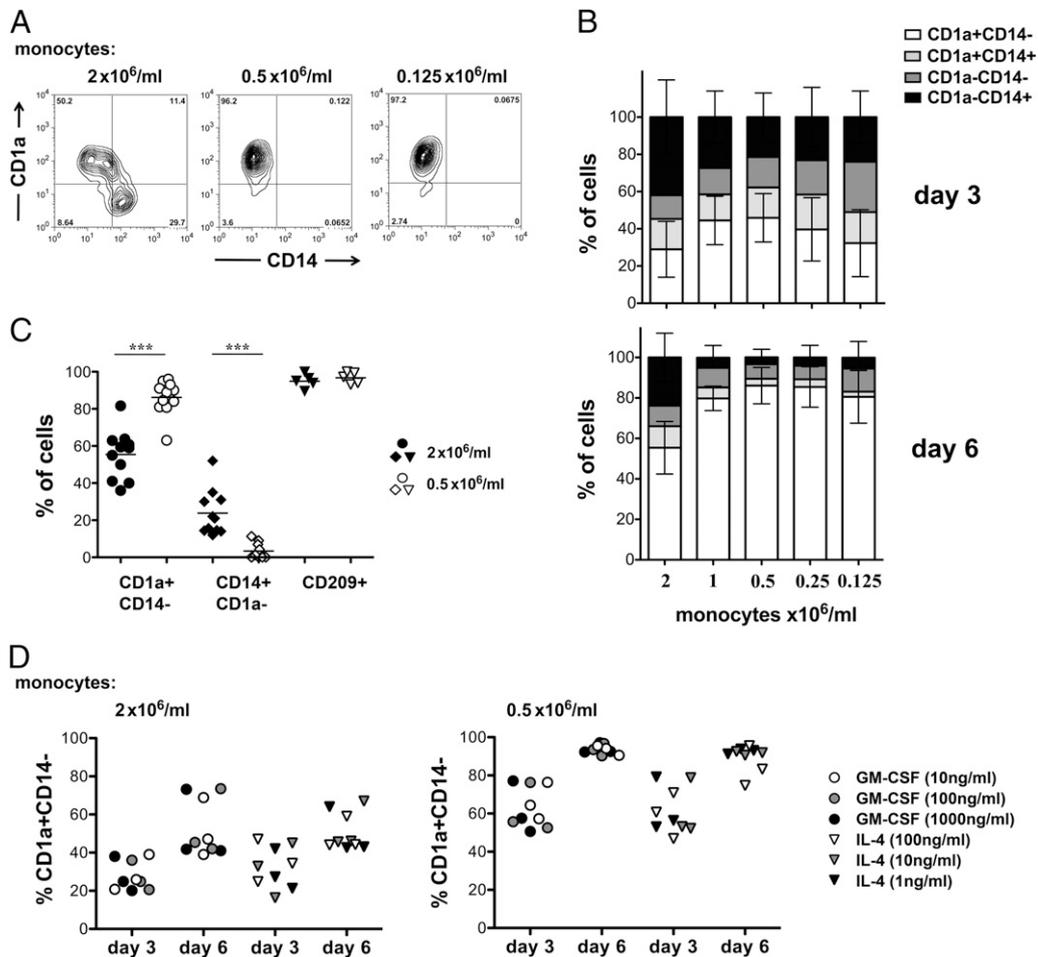
### Statistical analysis

Statistical analyses (paired *t* tests) were performed using Prism (version 5.0a for Mac OS X, GraphPad Software, San Diego, CA).

## Results

### Cell culture density modulates MoDC differentiation

Little is known about how cell culture conditions can influence MoDC differentiation. We assessed the potential influence of cell concentration on MoDC differentiation by culturing monocytes using densities between  $2 \times 10^6$  and  $0.125 \times 10^6$  cells/ml and analyzing CD1a and CD209 induction and CD14 downregulation. Interestingly, we observed high frequency of the CD14<sup>+</sup>CD1a<sup>-</sup> MoDC population when using the cell concentration of  $2 \times 10^6$  cells/ml, whereas, at lower densities, the monocytes differentiated to a rather homogenous population of CD1a<sup>+</sup>CD14<sup>-</sup> MoDCs (Fig. 1A). We analyzed the distribution of MoDCs in the CD1a<sup>+</sup>CD14<sup>-</sup>, CD1a<sup>+</sup>CD14<sup>+</sup>, CD1a<sup>-</sup>CD14<sup>-</sup>, and CD1a<sup>-</sup>CD14<sup>+</sup> populations in cultures that were established using different culture densities at days 3 and 6 of the culture ( $n = 11$ ; Fig. 1B). By day 3, the highest ratio of CD1a<sup>+</sup>CD14<sup>-</sup> cells was observed using the density of  $0.5 \times 10^6$ /ml, whereas both higher and lower densities resulted in a lower frequency of this subset (Fig. 1B, upper panel). By day 6, CD1a<sup>+</sup>CD14<sup>-</sup> MoDC proportion remained significantly lower using the density of  $2 \times 10^6$  monocyte/ml as compared with all other densities that allowed a rather homogeneous differentiation of CD1a<sup>+</sup>CD14<sup>-</sup> cells (Fig. 1B, 1C). Accordingly, the proportion of CD14<sup>+</sup>CD1a<sup>-</sup> MoDCs remained significantly higher using the density of  $2 \times 10^6$  monocyte/ml compared with all other densities (Fig. 1B, 1C). On the contrary to CD1a and CD14, CD209, another cell surface marker obtained during MoDC differentiation was present on >90% of MoDCs independently of the cell culture density ( $n = 5$ ), indicating that high cell culture density may not generally inhibit MoDC differentiation (Fig. 1C). These results suggested an unexpectedly strong influence of cell culture density on MoDC development; homogeneous CD1a<sup>+</sup>CD14<sup>-</sup> MoDC differentiation occurred at lower densities, whereas monocytes cultured at the density of  $2 \times$



**FIGURE 1.** Cell culture density modulates MoDC differentiation. Peripheral blood monocytes were cultured using different cell culture densities in the presence of GM-CSF and IL-4 and the expression of CD1a, CD14, and CD209 was analyzed by flow cytometry. **(A)** CD1a and CD14 expression at day 6 is shown in one representative experiment using the culture densities  $2 \times 10^6$ ,  $0.5 \times 10^6$ , and  $0.125 \times 10^6$  monocytes/ml, together with the gating strategy to identify the CD1a<sup>+</sup>CD14<sup>-</sup>, CD1a<sup>+</sup>CD14<sup>+</sup>, CD1a<sup>-</sup>CD14<sup>+</sup>, and CD1a<sup>-</sup>CD14<sup>-</sup> MoDC populations. The frequency of these subsets was analyzed at days 3 and 6 of the MoDC culture **(B)**. The diagrams are composed from mean values calculated from 11 independent experiments, SD is indicated for the CD1a<sup>+</sup>CD14<sup>-</sup> and CD14<sup>+</sup>CD1a<sup>-</sup> populations. **(C)** The frequency of the CD1a<sup>+</sup>CD14<sup>-</sup>, the CD14<sup>+</sup>CD1a<sup>-</sup> ( $n = 11$ ), and the CD209<sup>+</sup> ( $n = 5$ ) populations are shown for the densities of  $2 \times 10^6$  and  $0.5 \times 10^6$ , measured at day 6. Symbols represent the proportion of the different subsets in independent experiments ( $***p < 0.001$ ). **(D)** Monocytes were cultured in the presence of GM-CSF and IL-4 using the cell culture density of  $0.5 \times 10^6$  and  $2 \times 10^6/\text{ml}$  for 6 d. GM-CSF concentrations were 10, 100, or 1000 ng/ml (in combination with 50 ng/ml IL-4), and IL-4 concentrations were 1, 10, or 100 ng/ml (in combination with 75 ng/ml GM-CSF). At days 3 and 6, the percentages of CD1a<sup>+</sup>CD14<sup>-</sup> MoDCs were determined by flow cytometry.

$10^6$  cells/ml differentiated into a mixture of CD1a<sup>+</sup>CD14<sup>-</sup> and CD1a<sup>-</sup>CD14<sup>+</sup> cells. To test whether the cell culture density influenced DC development because of a different level of competition for GM-CSF or IL-4 in dense and sparse cultures, we compared MoDC differentiation using a wide range of cytokine concentrations. We detected a comparable development of CD1a<sup>+</sup>CD14<sup>-</sup> MoDCs using GM-CSF in the range of 10–1000 ng/ml or IL-4 in the range of 1–100 ng/ml, tested both at the density of  $2 \times 10^6$  and  $0.5 \times 10^6$  cells/ml, indicating that the altered MoDC development in dense or sparse cultures was not the consequence of variable cytokine availability (Fig. 1D).

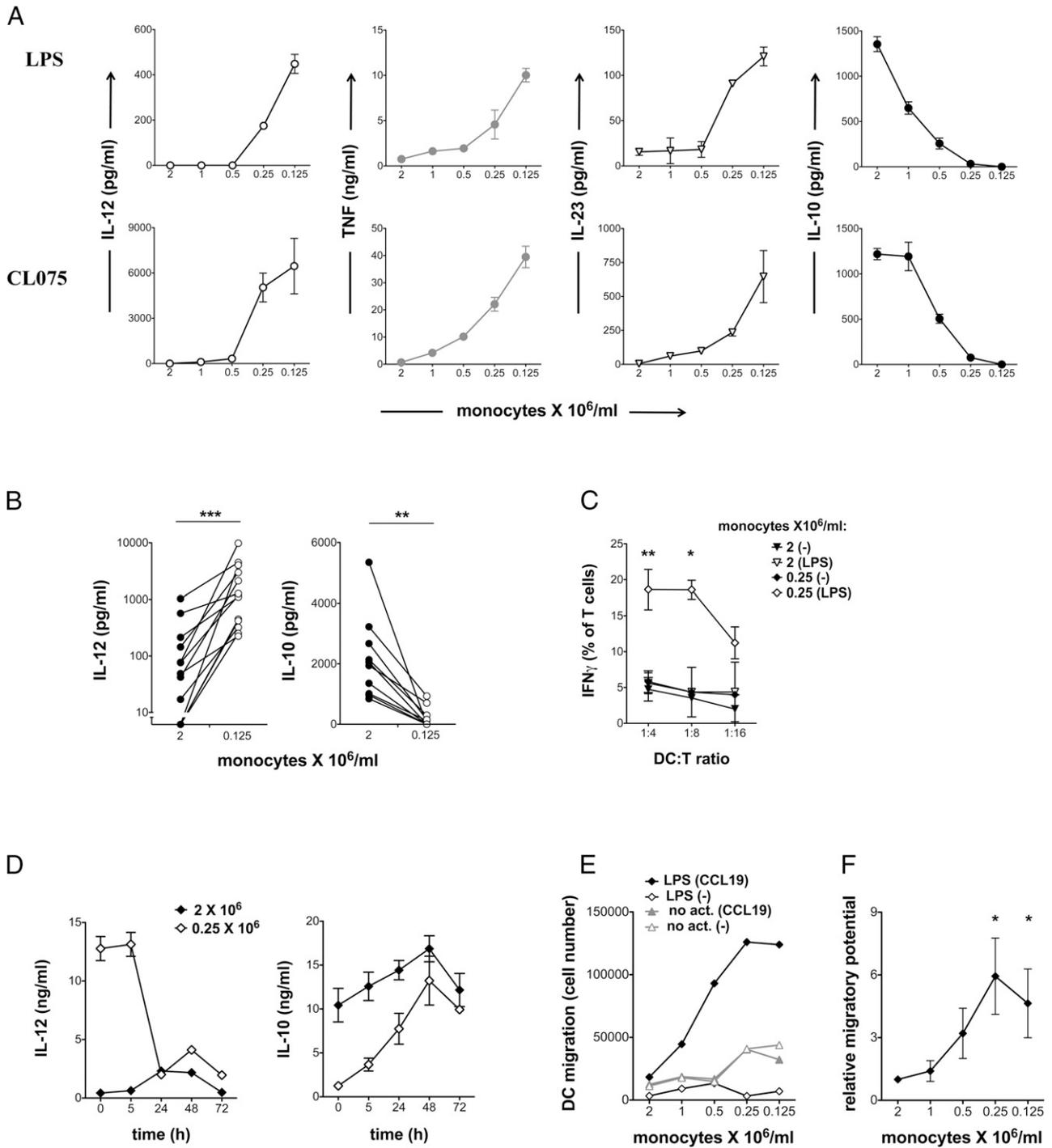
*Low cell culture density promotes inflammatory cytokine production; dense culture leads to IL-10 production*

MoDCs that developed at different culture densities were collected at day 6, recultured at equal densities and were activated by LPS for 24 h. We observed a comparable upregulation of CD83, CD86 and HLA-DQ on cells that previously differentiated at different cell culture densities indicating functional TLR4 signal in the various DC types (Supplemental Fig. 1A; data not shown). However, when production of the inflammatory cytokines TNF, IL-12, and IL-23

and the levels of IL-10 were compared between MoDCs that previously developed at different culture densities, we observed a very strong influence of cell concentration on cytokine production. MoDCs cultured at  $2 \times 10^6/\text{ml}$  density secreted high levels of IL-10, but very low levels of inflammatory cytokines upon stimulation by LPS or by the TLR7/8 ligand CL075 (Fig. 2A, 2B). Decreasing cell culture density eliminated most of the IL-10 production and resulted in high levels of TNF, IL-12, and IL-23 (Fig. 2A, 2B).

The results suggest that the functional properties of the developing MoDCs were more sensitive to culture density than the alteration of the phenotype, where loss of IL-12p70, TNF, and IL-23 and the increase of IL-10 already occurred at low density ( $0.5 \times 10^6$ ; Fig. 2A), whereas acquisition of the CD1a<sup>-</sup>CD14<sup>-</sup>CD209<sup>+</sup> phenotype was impaired only in the highest cell culture density (Fig. 1A, 1B).

Although we detected a remarkable variability in cytokine levels between experiments (Fig. 2B), the average IL-12 production was ~13-fold higher and the average IL-10 production was ~10-fold lower in LPS-activated MoDCs that were generated using the cell concentration of  $0.125 \times 10^6/\text{ml}$  as compared with  $2 \times 10^6/\text{ml}$ .



**FIGURE 2.** Cytokine production and migratory responses of MoDCs generated in dense or sparse cultures. Peripheral blood monocytes were cultured using different cell culture densities for 6 d with GM-CSF and IL-4, and thereafter, the cells were collected, cultured at equal  $0.5 \times 10^6$  cell/ml density and activated using LPS or CL075 for 24 h. **(A)** IL-12, TNF, IL-23, and IL-10 levels in the culture supernatants were compared between MoDCs that were generated using different cell culture densities. Representative results of at least three independent experiments are shown; mean values ( $\pm$  SD) were calculated from sample triplicates. **(B)** Levels of IL-12 and IL-10 are shown in the supernatants of LPS-activated MoDCs that were generated using the cell culture density of  $2 \times 10^6$  and  $0.125 \times 10^6$  monocytes/ml in 13 (IL-12) and 10 (IL-10) independent experiments. **(C)** MoDCs were generated using the density of  $2 \times 10^6$  or  $0.25 \times 10^6$  monocytes/ml. Thereafter, the cells were activated for 1 h with LPS, then washed and cultured with allogeneic naive CD4<sup>+</sup> T lymphocytes using different DC:T cell ratios. IFN- $\gamma$  production of T cells was analyzed on day 8, following a 4-h PMA/ionomycin treatment using intracellular flow cytometry. Mean frequency ( $\pm$  SD) of the IFN- $\gamma$ -expressing T cells was calculated from three independent experiments, and significant differences were observed comparing cytokine induction in the presence of LPS-pretreated DCs obtained from dense or sparse cultures. **(D)** MoDC cultures were maintained for 6 d using the cell culture densities of  $2 \times 10^6$  and  $0.25 \times 10^6$  cells/ml (0-h time point). Alternatively, cells differentiating in high-density cultures were collected 5, 24, 48, or 72 h after the initiation of the culture, washed, and recultured using the original volume (black symbols) or 8 $\times$  diluted (white symbols) until day 6. Then the cells were collected, recultured in equal,  $0.5 \times 10^6$  cell/ml density and activated using LPS. IL-12 and IL-10 production is shown in one out of three independent experiments; mean values ( $\pm$  SD) were calculated from triplicate samples. **(E)** MoDCs that were generated using different cell culture densities were collected on day 6 and were placed in the upper wells of Transwell plates in the presence or absence of 250 ng/ml LPS for 8 h. The ability of the cells to migrate through the membrane toward empty medium or 50 ng/ml CCL19 was (Figure legend continues)

Accordingly, low-density MoDCs were much more potent inducers of Th1 differentiation as compared with high-density MoDCs (Fig. 2C). IL-17, IL-4, and IL-10 were detected in <1% of the cells in these experiments, indicating the inefficiency of the MoDC-naive CD4<sup>+</sup> T cell coculture system to induce Th2 or Th17 differentiation, the latter in line with published data (17). These results suggest that the decreased MoDC culture density facilitates mostly Th1 differentiation, although we cannot exclude an effect on other Th lineages in other experimental settings.

Our results indicated the strong influence of cell concentration during MoDC differentiation on the later cytokine production of the cells. To determine whether the different densities induced a long-lasting functional programming in the developing MoDCs or only temporary alterations in cytokine production, we performed a sequential dilution experiment using the initial density of  $2 \times 10^6$  monocytes/ml and diluting samples to  $0.25 \times 10^6$  cells/ml 5, 24, 48, or 72 h later. Thereafter, we left the cells in culture until day 6. Interestingly, within the first 48 h, the low IL-12 and high IL-10-producing ability was permanently established in dense cultures, indicating a long-lasting functional programming of MoDCs in dense cultures (Fig. 2D).

#### *Increased migratory potential of low-density MoDCs toward CCL19*

Mobility of the in vitro generated MoDCs is a particularly important characteristic from a vaccine point of view; yet, previous reports indicated only a modest ability of the cells to leave the injection site in vaccinated individuals (6, 7). We found a comparable upregulation of the chemokine receptors CCR7 and CXCR4, specific for lymphoid tissue chemokines, and downregulation of CCR5 that is normally expressed on immature DCs when we activated MoDCs that developed at different cell culture densities (Supplemental Fig. 1B, 1C). In contrast, the cell culture density strongly influenced the migration toward the CCR7 ligand chemokine CCL19 (Fig. 2E, 2F). MoDCs that developed in sparse cultures migrated in high numbers following LPS-mediated activation in a Transwell system, whereas LPS-treated MoDCs obtained from dense cultures did not increase their migration toward CCL19 as compared with spontaneous movement (in the absence of chemokine) or to cell counts observed in the case of nonactivated DCs lacking CCR7 (Fig. 2E, 2F).

#### *Endogenous lactic acid inhibits inflammatory DC differentiation*

Day 2 supernatants of high-density MoDC cultures possessed a strong inhibitory effect on CD1a<sup>+</sup>CD14<sup>-</sup> DC development when added to sparse cultures (Supplemental Fig. 2). Using a m.w.-based filtration we observed the complete preservation of such inhibitory effects in the supernatant fraction containing molecules smaller than 3KD (Supplemental Fig. 2), suggesting that the developing MoDCs might release a small, non-protein type mediator that would accumulate in a density-dependent manner and might inhibit inflammatory DC differentiation. One possible candidate molecule was lactate, produced by cells that rely on glycolytic energy production (18). It is not known whether the developing DCs use glycolysis for their ATP production, however, when lactic acid is produced by tumors, it strongly inhibits inflammatory MoDC development (9). We analyzed lactate concentration in MoDC cultures at day 1, 3, or 6 of differentiation using different cell

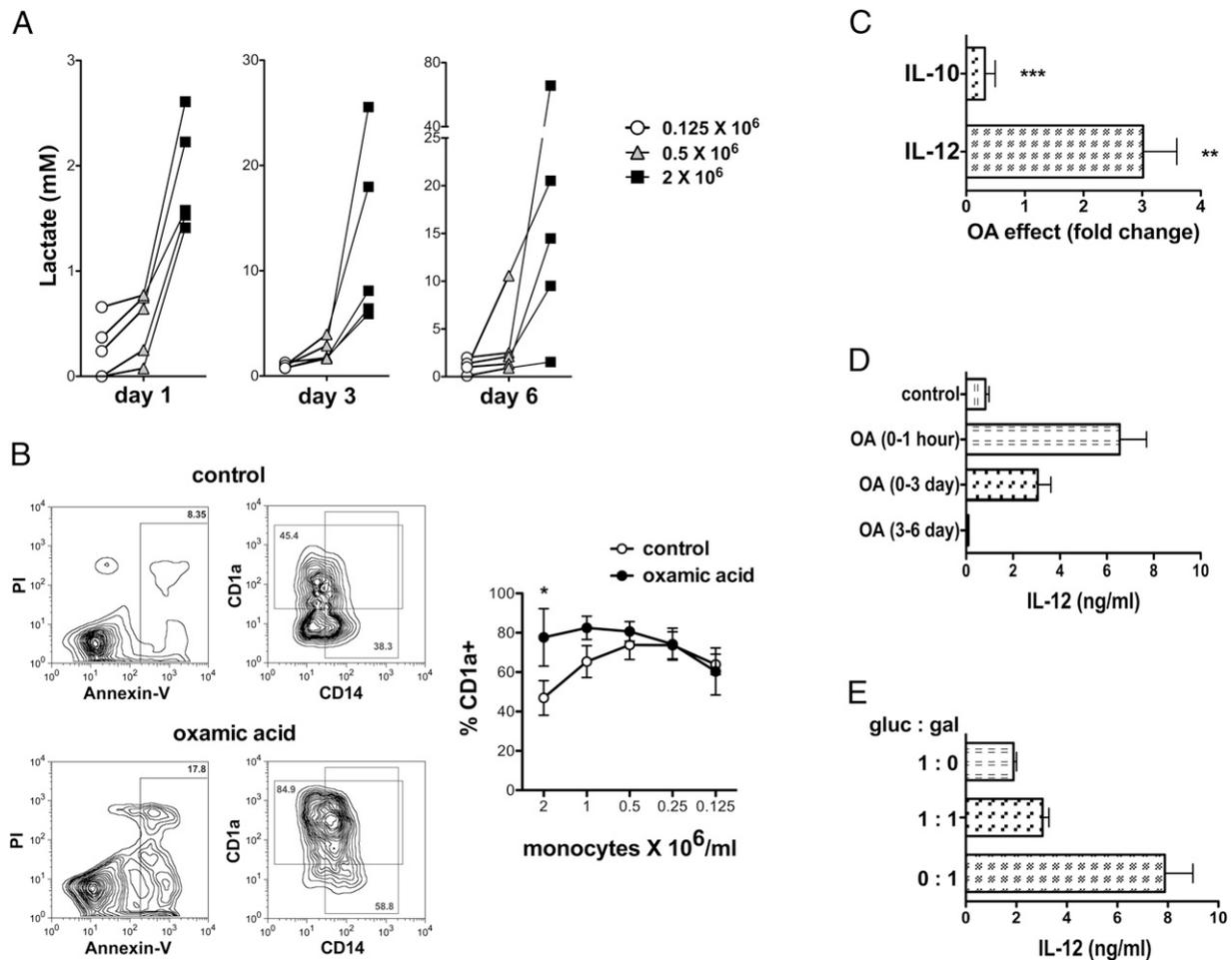
concentrations. We observed a massive lactate accumulation in dense MoDC cultures (Fig. 3A) and, importantly, the lactate levels in dense cultures were similar than in tumor cell cultures (9) where lactic acid strongly inhibited the differentiation of IL-12-producing CD1a<sup>+</sup> DCs.

In the presence of oxamic acid, an inhibitor of lactate dehydrogenase and lactic acid production, CD1a expression increased in high-density cultures, whereas in line with the very low lactate concentration observed at low culture densities, no effect was observed in sparse cultures (Fig. 3B). To compensate for the ~10% increase of apoptosis induced by oxamic acid (Fig. 3B, left panel), cell numbers in oxamic acid treated and untreated cultures were equalized before further functional assays. Importantly, MoDCs that developed in the presence of oxamic acid increased their IL-12 production ( $3.0 \pm 0.6$ -fold [mean  $\pm$  SD],  $n = 5$ ) and decreased their IL-10 production ( $4.3 \pm 2.9$ -fold [mean  $\pm$  SD],  $n = 5$ ) in response to LPS (Fig. 3C). CD14 expression increased also in the presence of oxamic acid indicating that CD14 downregulation in this case did not follow the differentiation of CD1a<sup>+</sup> IL-12-producing cells. In line with the early programming of the cytokine-producing abilities (Fig. 2D), 1-h pretreatment of monocytes with oxamic acid before the initiation of the MoDC cultures was sufficient to increase the LPS-induced IL-12 production on day 6, and later treatments (day 3) did not increase but rather eliminated IL-12 production (Fig. 3D), potentially because of the increasing importance of glycolytic ATP production in activated DCs (19). Despite the early removal of oxamic acid from the cell cultures we detected  $50 \pm 16\%$  reduction of lactic acid levels in dense cultures at day 3, as compared with untreated cultures ( $n = 3$ ) suggesting a long lasting effect of lactate dehydrogenase inhibition on glycolytic activity. Replacing glucose in the RPMI 1640 medium with galactose, a carbohydrate that cannot be used for glycolytic energy production, during the first 3 d of MoDC differentiation strongly increased IL-12 production in response to LPS on day 6, further indicating that a suppressed rate of glycolysis during the early phases of DC development facilitates IL-12 production (Fig. 3E). Complementing sparse MoDC cultures with lactic acid inhibited CD1a<sup>+</sup>CD14<sup>-</sup> MoDC development (Fig. 4A), decreased the LPS-induced IL-12 and increased IL-10 production (Fig. 4B). At neutral pH the lactate effect on CD1a<sup>+</sup>CD14<sup>-</sup> DC differentiation decreased strongly (Fig. 4A), whereas IL-12 remained inhibited (Fig. 4B). Acidity alone influenced modestly the differentiation markers or cytokines. Lactate and low pH thus provide a potent DC regulatory signal when present together, potentially as the consequence of the lactate and proton cotransportation system in the cell membrane driven by the concentration gradient of both compounds (20).

#### *Similar peroxisome proliferator-activated receptor $\gamma$ activity in sparse and dense MoDC cultures*

The intracellular lipid receptor molecule peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) has been repeatedly implicated in non-inflammatory MoDC differentiation (12, 21–26). This pathway is active in developing MoDCs due to PPAR $\gamma$  ligands being available in the serum or produced and secreted by the MoDCs (12, 26, 27). We tested the effects of the 15-lipoxygenase (15-LOX) enzymes that can generate PPAR $\gamma$  binding fatty acids, and PPAR $\gamma$  on MoDC differentiation in dense cultures using specific inhibitors for these molecules, Baicalein and GW9662 respectively (Fig. 5A).

tested via counting the cells in the lower chambers. Representative results of three independent experiments are shown or alternatively (F) LPS-induced migratory response was related to MoDCs originated from the highest ( $2 \times 10^6$  cells/ml) cell culture densities. Mean values ( $\pm$  SEM) were calculated from three independent experiments. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ .



**FIGURE 3.** Lactate accumulates in MoDC cultures in a density-dependent manner and regulates DC differentiation. **(A)** MoDC cultures were established using the cell culture densities of  $2 \times 10^6$ ,  $0.5 \times 10^6$ , and  $0.125 \times 10^6$  cells/ml in the presence of GM-CSF and IL-4, and lactate concentrations were measured in the supernatants on days 1, 3, or 6. Results obtained from several independent experiments are shown. **(B)** MoDC cultures were established using different cell culture densities in the presence of oxamic acid (1 mg/ml), and the drug-induced apoptosis as well as the CD1a and CD14 expressions were analyzed on day 3. The contour plots show Annexin V and PI binding as well as CD1a and CD14 expressions at the density of  $2 \times 10^6$  cells/ml in one representative experiment in the presence or absence of oxamic acid (OA), the diagram shows mean proportions of CD1a+ cells ( $\pm$  SD) calculated from three different experiments. **(C)** MoDCs, cultured at the density of  $2 \times 10^6$  cells/ml in the presence or absence of oxamic acid for 6 d, were collected, recultured in the presence of 250 ng/ml LPS for 24 h using the cell culture density of  $10^6$  cells/ml, and the levels of IL-12 and IL-10 in the culture supernatants were analyzed. Cytokine levels measured in OA-treated cells were normalized with the cytokine production of MoDCs that developed in the absence of oxamic acid. Fold changes in cytokine expression ( $\pm$  SD) were calculated from five independent experiments. **(D)** Oxamic acid was applied as a 1-h pretreatment, during the first 3 d of the MoDC culture or between days 3 and 6 to dense MoDC cultures ( $2 \times 10^6$  cells/ml). At day 6, the cells were collected and recultured in the presence of 250 ng/ml LPS for 24 h. IL-12 levels in the supernatants were analyzed using ELISA. Representative results of three independent experiments are shown; mean values ( $\pm$  SD) were calculated from triplicate samples. **(E)** Glucose was fully or 50% replaced in the RPMI 1640 medium during the first 3 d of the MoDC culture with galactose. IL-12 production of the cells was analyzed on day 6, following LPS activation. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ .

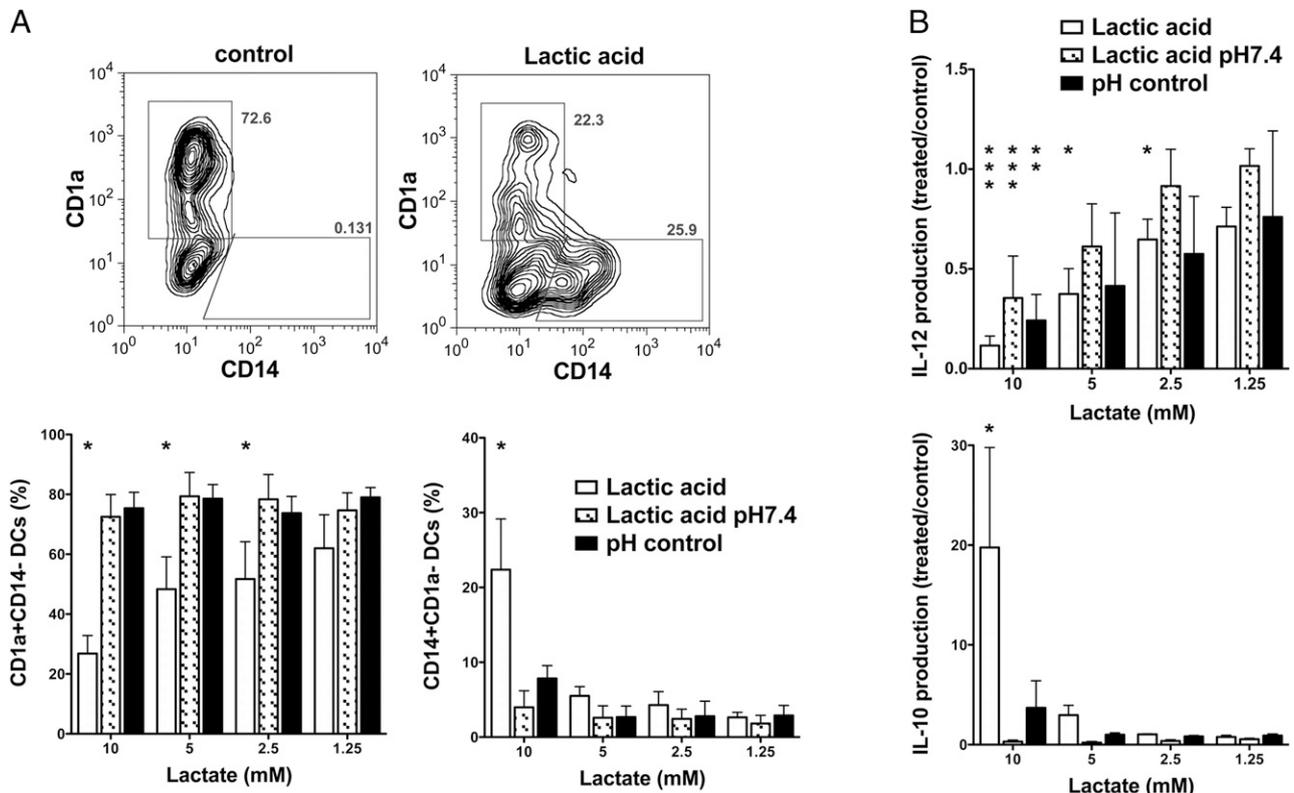
The significant increase in LPS-induced IL-12 production in the case of MoDCs that developed in the presence of the inhibitors ( $p < 0.05$  for both Baicalein and GW9662,  $n = 8$ ) indicates the steady state 15-LOX and PPAR $\gamma$  activity in differentiating MoDCs, as well as their inhibitory effect on IL-12 production.

Several compounds generated via linoleic or arachidonic acid oxidation bind PPAR $\gamma$ , including 9- and 13-HODE, 12- and 15-HETE and 15-KETE; however, other related fatty acid derivatives may act similarly. To understand whether the developing MoDCs produce PPAR $\gamma$  stimulatory compounds differentially in dense and sparse cultures, we performed a lipid metabolic profiling (16) in day-2 MoDCs and in their supernatants using the cell culture densities  $2 \times 10^6$ ,  $0.5 \times 10^6$ , or  $0.125 \times 10^6$  monocytes/ml. Fig. 5B shows that the amount of multiple arachidonic acid derivatives increased by day 2 in MoDCs compared with monocytes and we detected both linoleic and arachidonic acid-derived molecules in

the supernatants. However, none of the detected compounds showed a preferential accumulation in either sparse or dense cultures. The lack of any effect of cell culture density (16 $\times$  difference between highest and lowest density) on the concentrations of the detected compounds in the supernatant suggests that these molecules are probably not produced or consumed significantly during MoDC development. A snapshot analysis at day 2 thus indicated that the density-dependent DC regulation is not operating via PPAR $\gamma$ . Studying the expression kinetics of a wide range of PPAR $\gamma$  target genes (24–26) at different culture densities confirmed this hypothesis as most of the studied target genes were expressed at similar or lower level in dense versus sparse cultures (Fig. 5C).

#### MoDC in dense cultures can differentiate into osteoclasts

MoDCs obtained from high-density cultures showed very little ability to produce inflammatory cytokines, to trigger Th1 differentiation



**FIGURE 4.** Lactic acid inhibits inflammatory DC development. **(A)** L-Lactic acid was applied to MoDCs cultured at the density of  $0.25 \times 10^6$  cells/ml in different concentrations and the expression of CD1a and CD14 was analyzed on day 6 of the culture. Contour plots indicate the effect of 10 mM lactic acid on CD1a and CD14 expression in one representative experiment; the diagrams show average frequencies ( $\pm$  SEM) of CD1a<sup>+</sup>CD14<sup>-</sup> or CD1a<sup>-</sup>CD14<sup>+</sup> MoDCs using different lactic acid concentrations without pH adjustment, with pH adjusted to 7.4, or alternatively with pH decreased to the same level as in the presence of lactic acid using HCl. **(B)** With the same experimental procedure, the effect of lactic acid on the LPS-induced IL-12 and IL-10 secretion is shown. Mean values ( $\pm$  SEM) were calculated from three independent experiments. Values were compared with untreated controls: \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ .

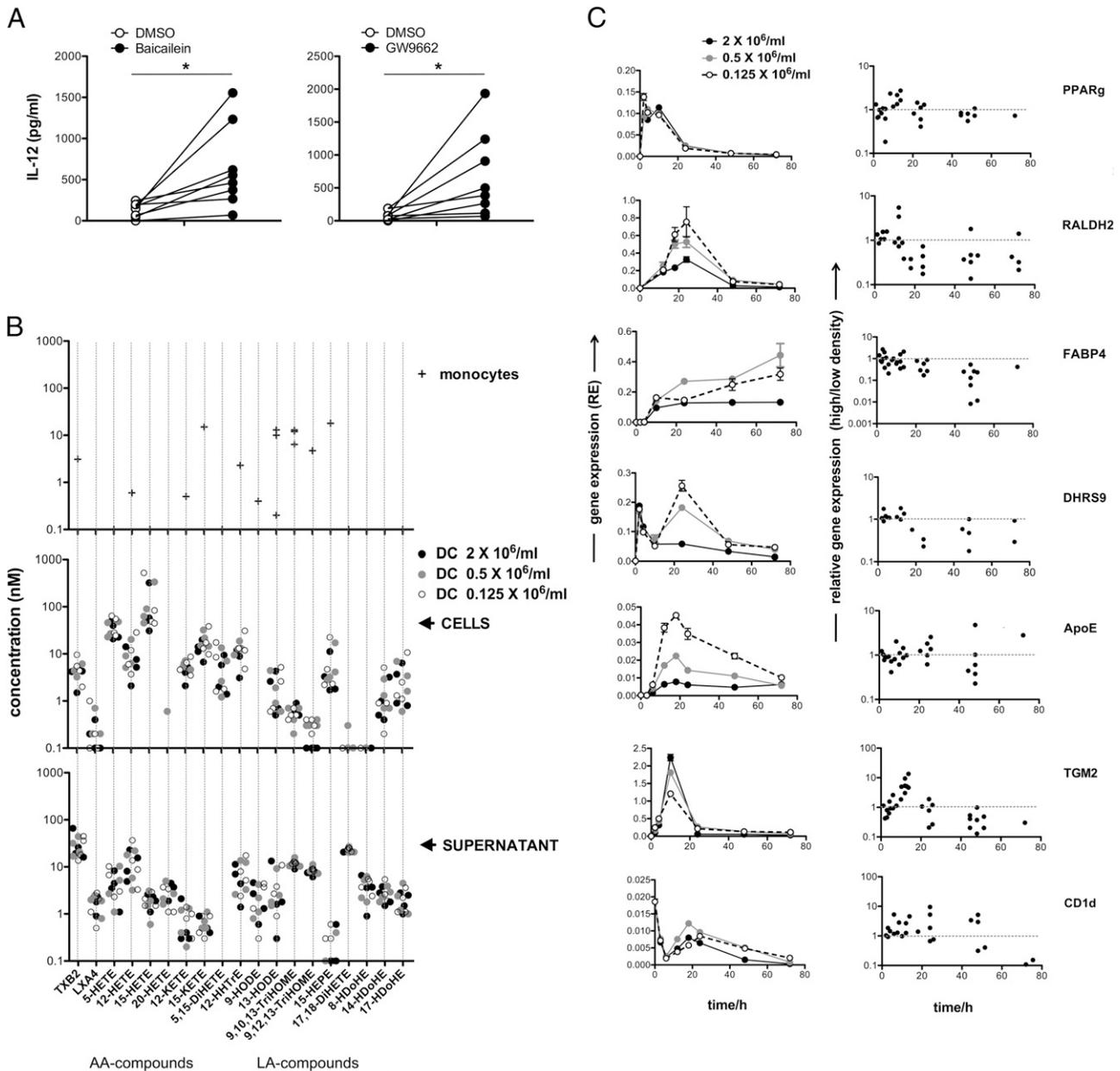
or to migrate toward CCL19. Nevertheless, these cells produced IL-10 upon activation (Fig. 2), suggesting that high cell culture density does not suppress all cellular functions but might promote alternative, noninflammatory differentiation pathways. To analyze whether the density-dependent inhibition of inflammatory DC development could lead to altered differentiation plasticity, we compared the ability of MoDCs that were generated at different cell culture densities to form osteoclasts, a cell type involved in bone homeostasis. We recultured day-6 MoDCs in the presence of MCSF and RANK-L, cytokines that promote osteoclast differentiation (28). Two weeks later, multinucleated ( $\geq 3$  nuclei) cells, positive for tartrate-resistant acid phosphatases (TRAP) that are enzymatic markers for osteoclasts and their precursors, were observed in case of all cell culture densities; however, osteoclast numbers were much higher when MoDCs derived from high-density conditions were used as precursors (Fig. 6A, B). Accordingly, osteoclasts that originated from high-density MoDCs possessed superior bone resorption activity as compared with MoDCs from low-density cultures (Fig. 6C, 6D). MoDC that differentiated in dense cultures following a 1-h pretreatment with oxamic acid or in the presence of galactose instead of glucose during the first 3 d of the culture were less efficient to develop functional osteoclasts as compared with their untreated counterparts indicating a role for aerobic glycolysis during MoDC development in potentiating the *trans*-differentiation of the cells to osteoclasts (Fig. 6E). The density-dependent retention of the developing cells from the inflammatory MoDC pathway was thus associated with an increased differentiation ability to another cell type, osteoclast. These results, together with the density-dependent increase of IL-10 production, indicate that the difference between

dense and sparse cultures are not simply quantitative, that is, DCs would be less functional in dense cultures, but that different culture densities can induce unique differentiation pathways in monocytes.

## Discussion

MoDCs that differentiate in the presence of GM-CSF and IL-4 can be surprisingly heterogeneous, as we demonstrated in the current study, acting either as inflammatory cells that promote TH1 polarization or obtaining a non-inflammatory phenotype, with the differentiation choice predominantly determined by the cell culture density. We showed that MoDCs produce lactic acid during their development that accumulates in dense cultures, inhibiting CD1a<sup>+</sup>CD14<sup>-</sup> MoDC differentiation and promoting IL-10 but no IL-12 production in response to TLR stimuli. In addition, MoDCs preserved higher differentiation plasticity in dense cultures, as demonstrated by their ability to *trans*-differentiate into osteoclasts. Decreasing cell culture densities eliminated lactate accumulation in the supernatants and increased the commitment to inflammatory DC differentiation. MoDCs from sparse cultures produced very high levels of IL-12, TNF and IL-23 upon activation and migrated efficiently toward the lymphoid tissue chemokine CCL19.

On the basis of our knowledge, glucose metabolic pathways that are active during MoDC differentiation remained unnoticed in DC-based research and therapies, despite their instrumental effects on key DC functions observed in the current study. Ex vivo generated DC vaccines might affect immune responses according to a functional program obtained in early stages of differentiation in a cell culture density dependent manner. We showed that the inhibitory effects of lactic acid on inflammatory cytokine production, Th1

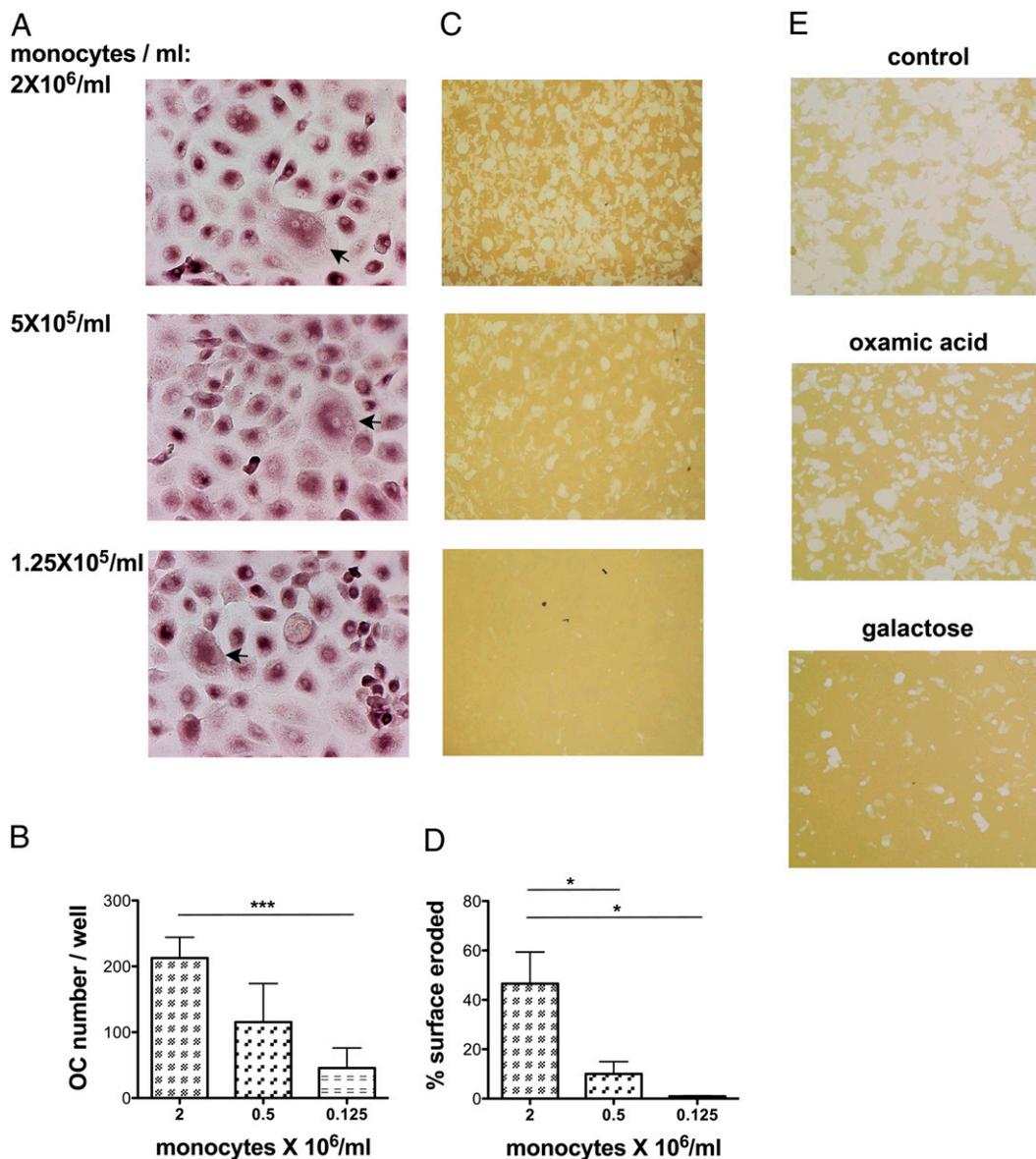


**FIGURE 5.** PPAR $\gamma$  activity in dense and sparse MoDC cultures. **(A)** MoDC cultures were established using the cell density of  $2 \times 10^6$  cells/ml in the presence of the 15-LOX inhibitor Baicalein, the PPAR $\gamma$  inhibitor GW9662 or with DMSO control. At day 6, the cells were activated with LPS for 24 h, and the production of IL-12 was analyzed in the cell culture supernatants. Results of eight independent experiments are shown (\* $p < 0.05$ ). **(B)** Monocytes were cultured in the presence of GM-CSF and IL-4 for 2 d using the cell concentrations of  $2 \times 10^6$ ,  $0.5 \times 10^6$ , and  $0.125 \times 10^6$  cells/ml ( $n = 4$ ). Concentrations of a range of lipid mediators (Supplemental Table I) were determined in monocytes, in day 2 MoDCs and day 2 MoDC supernatants using mass spectrometry. Concentration of the compounds that could be quantified in at least two different donors out of four are shown in the extracts prepared from  $0.5 \times 10^6$  pelleted cells or 500  $\mu$ l supernatants. **(C)** The expression kinetics of PPAR $\gamma$  target genes was analyzed in MoDC cultures established using the cell culture densities of  $2 \times 10^6$ ,  $0.5 \times 10^6$ , and  $0.125 \times 10^6$  cells/ml. Relative expression values were obtained following the normalization of PPAR $\gamma$  target gene expressions with the expression of the 36B4 housekeeping gene in all samples. Results of a representative experiment are shown on the left panel, or alternatively, gene expressions measured in dense cultures ( $2 \times 10^6$  cells/ml) were compared with expression levels observed in low-density cultures ( $0.125 \times 10^6$  cells/ml). Results were included from at least five independent experiments (right panel).

polarization and CCL19-driven migration were absent in sparse cultures. Therefore, it is tempting to speculate that MoDCs from sparse cultures might possess a superior anti-tumor activity due to their migratory potential and ability to mount tumor-specific Th1-type responses and provide inflammatory cytokines that can potentially counteract the immunosuppressive tumor milieu.

Endogenous lactate production has not been studied during DC differentiation, however, lactate is known to serve as an important signaling mediator in tumors. Proliferating malignant and non-malignant cells increase glycolytic activity to produce ATP and, at the

same time, to preserve intermediary products of the glucose metabolic pathways for new molecule synthesis (18). Increased glycolysis leads to lactic acid production and release via proton-linked monocarboxylate transporters driven by a lactate and proton gradient between the extra- and intracellular spaces (20). Lactic acid production by tumors decreases glycolysis, regulates hypoxia inducible factor-1 $\alpha$  activity and cellular motility and extracellular matrix production of nearby fibroblasts (20, 29). Importantly, lactic acid production of tumors can also inhibit the differentiation of CD11a<sup>+</sup> IL-12-producing DCs potentially inhibiting tumor-specific T cell responses (9).



**FIGURE 6.** MoDC originated from dense cultures show high potential to differentiate into osteoclasts. MoDCs generated using different cell culture densities were collected at day 6 and recultured in equal numbers in the presence of M-CSF and RANK-L for 14 d. Multinucleated ( $\geq 3$  nuclei), TRAP<sup>+</sup> cells (marked by arrow) were counted in the different cultures, representative images (**A**) (original magnification  $\times 250$ ) or mean numbers ( $\pm$  SEM) calculated from three independent experiments (**B**) are shown. Osteoclasts, derived from MoDCs that were generated using different cell culture densities, were tested for their bone-resorptive ability on a synthetic calcium phosphate coated surface. Representative images (**C**) (original magnification  $\times 40$ ) or mean surface erosion ( $\pm$  SEM) calculated from three independent experiments (**D**) are shown ( $*p < 0.05$ ,  $***p < 0.001$ ). Aerobic glycolysis was suppressed in high cell culture density MoDCs cultures using 1-h pretreatment of the monocytes with oxamic acid or by replacing glucose with galactose in the first 3 d of the MoDC culture. Thereafter, osteoclasts were generated from these MoDCs, and their bone resorptive potential was analyzed (**E**) (original magnification  $\times 40$ ). Representative images from two independent experiments are shown.

The reasons underlying the high glycolytic activity of the developing MoDCs and the consequent lactic acid secretion are yet to be understood. Aerobic glycolysis is an unlikely pathway of ATP production for nonproliferating cells (18). In addition, non-activated DCs have been reported to rely on mitochondrial respiration (30, 31); however, in light of our findings, these results might be specific for sparse cultures only. In LPS-activated murine DCs, glycolytic energy production is known to increase, as a specific adaptation mechanism for the increased intracellular nitrogen oxide levels that inhibits mitochondrial respiration, which scenario may not be applicable for MoDC development (19, 32).

In developing MoDCs other mechanisms might be responsible for lactic acid production. First, the monocyte-DC transition might create a similar demand for new molecule synthesis as it is sug-

gested for proliferating cells, where the construction of new cells diverts nutrients from complete oxidation. In contrast, glucose metabolic pathways might be also affected by the altered extracellular milieu, the relocation of monocytes from serum to culture, which could possibly lead to the necessity of synthesizing several compounds by the cells from precursors available in the cell culture medium. DCs in sparse cultures might efficiently release lactic acid to the medium, due to the constant proton and lactate gradient between intra- and extracellular spaces. On the contrary, lactate release could be inhibited in dense cultures by extracellular lactate accumulation and the acidification of the medium. In addition, cells in dense cultures might rapidly decrease the availability of key nutrients that might lead to a density-dependent reprogramming of the metabolic pathways.

Our results indicate that the unexpectedly diverse MoDC functions, associated with dense or sparse MoDC cultures, might be programmed in the developing cells by lactic acid that accumulated in dense cultures. PPAR $\gamma$ , previously identified as a potent endogenous regulator of MoDC differentiation, inhibitory for both CD1a and IL-12 expression, had no effect on the density-dependent DC regulation. It will be important to see whether the modulation of glucose metabolic pathways could represent a feasible strategy to achieve an improved efficiency of therapeutic DC applications. Different cell culture densities, as we demonstrated in this study, can prime opposing DC activities via the different lactate levels, potentially influencing the interactions of DCs with other cells of the immune system as well as with microbes. Our results may indicate a strongly context-dependent nature of the in vitro MoDC model system, due to DC functions being already polarized in early stages of differentiation by cell culture density, but these results also provide the possibility for efficient and remarkably simple DC manipulation.

## Disclosures

The authors have no financial conflicts of interest.

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