RESEARCH ARTICLE

Ascorbic acid modulates cell migration in differentiated HL-60 cells and peripheral blood leukocytes

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Scope: The impact of L-ascorbic acid (L-AA) on the chemokinesis (CK) and chemotaxis (CT) of HL-60 cells and polymorphonuclear cells (PMN) was investigated.

Methods and results: HL-60 cells were differentiated with DMSO, retinoic acid (RA), vitamin D, or L-AA. Chemokinesis and chemotaxis of differentiated HL-cells were assayed. Vitamin D3-treated HL-60 cells (dHL-60$_{vitD3}$ cells) and RA-treated cells (dHL-60$_{RA}$ cells) acquired monocyte/macrophage-like and neutrophil-like phenotypes, respectively. DMSO induced the differentiation of an intermediate phenotype (dHL-60$_{DMSO}$ cells), whereas L-AA downregulated neutrophil markers (dHL-60$_{L-AA}$ cells). dHL-60$_{DMSO}$ cells had increased CK and potent CT in gradients of IL-8 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). dHL-60$_{RA}$ cells and dHL-60$_{L-AA}$ cells migrated less toward IL-8 and fMLP; dHL-60$_{vitD3}$ cells preferably responded to fMLP. L-AA enhanced CK of dHL-60$_{DMSO}$ cells and was a weak chemo-attractant. In human leukocytes, IL-8 and fMLP triggered receptor-mediated chemotaxis. CXCR2 and fMLPR were downregulated by IL-8 and fMLP, respectively. L-AA stimulated chemotaxis although significantly less than IL-8 and fMLP. IL-8 targeted chemotaxis was enhanced both in HL-60 cells and leukocytes when cells were incubated with L-AA.

Conclusion: L-AA modulated chemokinesis and had significant chemo-attractant properties, which were independent on fMLP or IL-8 receptors. The results suggest that L-AA improves leukocyte function in innate immune responses.

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1 Introduction

Among immune cells, neutrophils have a central role in the shaping of the immune response. The function of neutrophils in immunity and in vascular inflammation has been established in numerous studies [1]. Inflammation can be triggered by an infectious agent or by intravascular and sterile injury. Neutrophils sense systemic changes, migrate to affected tissues, and perform multiple tasks to restore homeostasis [2]. These processes are initiated and orchestrated by chemo-attractants (pathogen-derived products such as N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) or host-derived IL-8, leukotriene B4, and C5a), that recruit neutrophils to sites of inflammation along complex navigation patterns [3]. These events are mainly controlled by factors derived from pathogens and by inflammatory signals. Yet, the nutritional status of the host contributes to, and modulates the immune response. Specifically, the effects of micronutrients like vitamin A, vitamin D, and vitamin C (L-ascorbic acid, L-AA) on the adaptive and innate immune response have been investigated. L-AA, which is accumulated in leukocytes by an active transport mechanism [4], may facilitate oxidative destruction of bacteria by neutrophils, preserve neutrophil integrity, and protect tissues during the oxidative burst [5]. L-AA has been shown to restore or enhance neutrophil function in the Chediak–Higashi syndrome (CHS) [6–10] and increase

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Abbreviations: CHS, Chediak–Higashi syndrome; **CK**, chemokinesis; **CT**, chemotaxis; **fMLP**, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; **GM-CSF**, Granulocyte macrophage colony stimulating factor; **L-AA**, L-ascorbic acid; **PBL**, peripheral blood leukocytes; **PMN**, polymorphonuclear leukocytes; **PBMC**, peripheral blood mononuclear cells; **RA**, retinoic acid; **vitD3**, (25)OH vitamin D3

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complement-induced leukocyte movement [11–13]. In guinea pigs, neutrophil chemotaxis was reduced in animals fed a low L-AA diet [14]. The in vivo leukocyte functions in normal individuals or CSH patients supplemented with L-AA have been investigated [6, 9, 11, 15–17]. L-AA acts on the neutrophil microtubule network and thus promotes cell movement in CHS patients [18]. An improvement of chemotactic migration of neutrophils has been shown in some studies [19], whereas no effect was reported in others [20, 21].

In this study, we evaluate the effects of micronutrients such as L-AA on the phenotype and function of peripheral blood leukocytes (PBL) and HL-60 cells. Since L-AA modulate various immune function, validated immune markers that respond to L-AA intake and levels need to be identified. Blood leukocytes and their myeloid cell line homologues might serve as a surrogate model for responses in vivo and be appropriate to validate effects of nutrients in vitro. We have established a relationship between the function of PBL and the L-AA status. The data should be useful in assessing neutrophil functions as a biomarker for L-AA on immunity.

2   Materials and methods

2.1   Materials and reagents

Granulocyte/macrophage colony stimulating factor (GM-CSF), IL-8, and fMLP were from PeproTech (London, UK). Retinoic acid (RA), 25-hydroxyvitamin D3 (vitD3), and L-ascorbic acid (L-AA) were from Sigma (St. Louis, MO). Substances were dissolved in medium, DMSO or in ethanol as appropriate; L-AA solutions were adjusted to pH 7.4 with sodium carbonate. Cell culture media and reagents were from Invitrogen (Carlsbad, CA) and ATCC (Manassas, VA); 24-well Transwell tissue culture plates (with polycarbonate membranes, pore size 5 μm) were from Corning (Reinach, Switzerland); fluorochrome-conjugated monoclonal antibodies (mabs) and isotype control mabs were from BD Pharmingen (San Diego, CA) or eBioscience (Vienna, Austria). FITC-conjugated mabs against CD11b, CD18, CD93w, and CD181 were used; mabs against CD16b, CD88, fMLP receptor, CD14, CD18, CD116, and CD66c were conjugated with PE and mabs against CD11b, CD66, and CD182 were labeled with APC.

2.2   Cells

2.2.1   Peripheral blood leukocytes

Human in vitro peripheral blood leukocyte experiments were approved by the Swiss Federal Office of Public Health (No. A050573/2) and the Ethical Commission of the Kanton Aargau, Switzerland. Blood was collected from the antecubital vein and processed within < 1 h. PBL were isolated as detailed before; polymorphonuclear leukocytes (PMN) were separated from mononuclear cells (PBMC) as described [22].

2.2.2   HL-60 cells

HL-60 cells were obtained from ATCC and cultured in IMDM supplemented with 10% heat-inactivated FBS, streptomycin (50 μg/mL), penicillin (50 U/mL), HL-60 cells were differentiated with 1.25% DMSO, all-*trans* retinoic acid (RA, at 1 μmol/L) [23], 25(OH)vitamin D_3 (at 1 μmol/L), or with L-AA (at 50–1000 μmol/L) for 4–6 days.

2.3   Cytofluorometry

Washed cells were resuspended in HBSS/2% FCS/0.01% NaN_3 (HFN), and incubated with fluorochrome-conjugated antibodies for 45 min at 4°C. Subsequently, cells were washed in HFN, resuspended in HFN containing 7-AAD (2.5 μg/mL). Data were acquired with a FACS Calibur cytofluorometer and evaluated with Cellquest software (Becton & Dickinson, Mountain View, CA).

2.4   Chemotaxis and chemokinesis of peripheral blood leukocytes and HL-60 cells

Chemotaxis (CT) assays were performed in Transwell chambers (Corning, 5 μm pore size). Cells were pretreated with substances for 1–2 h prior to the assay. Cells were resuspended in CT medium (RPMI 1640 without phenol red, 20 mM HEPES pH 7.4, 0.5% BSA; NEAA, 50 μM 2-mercaptoethanol) and 2×10^6 cells were placed in the upper compartment. Chemo-attractants (fMLP; IL-8; GM-CSF; C5a; L-AA) were added to the lower compartments. Cell migration was allowed for 1 h at 37°C, except where stated differently. Chemokinesis (CK) was measured when chemo-attractants were placed in both compartments. Cells were counted with the MoxiZ® cell counter device (Orflo Technologies, Bothell, WA).

2.5   Determination of cellular ascorbic acid contents

Freshly isolated PBLs were lysed in 5% meta-phosphoric acid (MPA) and L-AA was determined as described [4]. Where indicated, cells were resuspended in PBS containing L-AA, incubated at room temperature (with gentle rotation), washed in PBS, lysed in MPA and the cellular L-AA was determined.

2.6   Statistical analysis

Data are presented as mean ± SD or ± SEM. The difference between means was assessed by the Student test. *p* values of <0.05 were considered to reflect statistically significant differences.

Table 1. Mean fluorescence intensity of surface determinants on peripheral blood leukocytes and HL-60 cells

CD	Common name	Lympho-cytes	Monocytes	PMN	Undifferentiated HL-60	DMSO-differentiated HL-60	Retinoic acid-differentiated cells	25(OH)vitamin D3 differentiated cells	L-AA differentiated cells
CD11b	MAC-1	59 ± 21	552 ± 24	558 ± 56	27 ± 15	51 ± 27	94 ± 20	175 ± 36	133 ± 2 1
CD14	LPS-receptor	25 ± 15	1354 ± 120	42 ± 11	63 ± 43	376 ± 183	86 ± 31	2732 ± 1858	77 ± 32
CD18	Integrin-b2	202 ± 12	1529 ± 321	645 ± 34	506 ± 59	418 ± 105	424 ± 122	808 ± 239	343 ± 47
CD66	CECAM	25 ± 3	34 ± 5	486 ± 121	41 ± 16	63 ± 31	357 ± 120	31 ± 11	36 ± 24
CD182	CXCR2	29 ± 10	48 ± 6	186 ± 27	71 ± 27	74 ± 18	94 ± 19	76 ± 23	112 ± 64
	fMLPR	19 ± 5	30 ± 18	20 ± 9	37 ± 13	52 ± 24	29 ± 8	35 ± 15	37 ± 17
CD116	GM-CSF receptor	21 ± 12	263 ± 23	38 ± 13	38 ± 10	92 ± 23	91 ± 8	121 ± 3	32 ± 4

Freshly isolated PBLs, or undifferentiated and differentiated HL-60 cells were stained with fluorochrome-conjugated antibodies and the mean fluorescence intensity determined by cytofluorometric analysis. Lymphocytes, monocytes, and PMN were gated according to their forward and side light scattering. Data are mean intensities ($\pm$SD) of at least three independent experimental series done in duplicate.

3 Results

3.1 Retinoic acid, vitamin D3, and DMSO induced distinct HL-60 cellular phenotypes

We incubated HL-60 cells with DMSO, vitD$_3$, or RA [23, 24] for 4 days and analyzed their phenotype by cytofluorometry. CD11b was expressed on <10% of undifferentiated HL-60 and was markedly upregulated by DMSO, RA, and vitD$_3$ (Table 1). CD18 expression was increased by vitD$_3$. Broad CD14 expression was observed on a subpopulation of dHL-60$_{RA}$ cells and dHL-60$_{DMSO}$ cells. CD14 density was highest on vitD$_3$-differentiated cells (dHL-60$_{vitD3}$), which adhered to plastic surfaces [25]. CD66 expression was increased in dHL-60$_{RA}$ cells and in a subpopulation of dHL-60$_{DMSO}$ cells (Supporting Information Fig. 1). dHL-60$_{DMSO}$ cells consisted of CD14$^+$/CD66$^-$ and CD14$^-$/CD66$^+$ cells, whereas dHL-60$_{vitD3}$ cells and dHL-60$_{RA}$ were CD14$^+$/CD66$^-$ and CD14$^-$/CD66$^+$, respectively. fMLPR was expressed on ~50% of dHL-60$_{DMSO}$ cells. CXCR2 was detected on differentiated HL-60 cells and it was increased by RA and L-AA (Table 1). Collectively, RA and vitD$_3$ induced a neutrophil-like and monocyte/macrophage-like phenotype, respectively. Physiological L-AA concentrations caused only minor differences in the HL-60 phenotype (Supporting Information Fig. 1 and Table 1).

3.2 Comparison of phenotypes of differentiated HL-60 cells and PBL

We compared the expression of leukocyte determinants on PBLs (lymphocytes, monocytes/macrophages, PMN) with that of differentiated HL-60 cells. Monocytes and PMN were brightly CD11b-positive. CD14 and CD18 determinants were most expressed on monocytes (Fig. 2, Table 1). CD66 determinants and CXCR2 were only detected on PMN. fMLPR was mainly present at the surface of monocytes and PMN (Fig. 2). GM-CSF receptors (CD116) were predominant on monocytes (Table 1). Expression levels of PMN surface

Table 2. Checkerboard analysis. (A) Checkerboard analysis for HL-60 cells. L-AA as chemo-attractants for dHL-60 $_{DMSO}$ cells.[a] (B) Checkerboard analysis for PBL. L-AA as chemo-attractants for PBL.[b]

L-AA in lower wells (μmol/L)	L-AA in upper wells (μmol/L)		
	0	50	200
A			
0	22.3 ± 0.8	25.3 ± 1.1*	24.3 ± 0.6*
50	23.2 ± 1.1	25.5 ± 1.2*	23.6 ± 0.7*
200	22.0 ± 1.1	25.0 ± 0.6*	21.7 ± 1.4
B			
0	20.5 ± 6.8	21.9 ± 0.7	16.8 ± 3.0*†
50	21.5 ± 3.1	24.05 ± 2.9*†	16.5 ± 2.0*
200	23.7 ± 0.6*	30.6 ± 5.2*†	19.7 ± 2.9†

a) dHL-60$_{DSMO}$ cells were incubated with L-AA in the upper and lower compartment at 37°C for 1 h. Results are the mean of triplicates of transmigrated cells ($\times 10^4$) ± SD. *p < 0.1 compared to 0/0 wells.
b) PBLs were incubated with L-AA in the upper and lower compartment at 37°C for 1 h. Results are the mean of triplicates of transmigrated cells ($\times 10^4$) ± SD. *p < 0.1 (versus 0/0 wells). † indicates p < 0.1 compared to L-AA in lower wells.

Table 3. Proportion of migrated cells

	Migration towards[a]	PMN	Monocytes	Lymphocytes
Total PBLs		69.5 ± 0.9[b]	12.9 ± 1.0	10.3 ± 0.5
Migrated cells	Medium	76.6 ± 1.4	0.4 ± 0.4	17.1 ± 0.8
	IL-8	88.7 ± 0.1	0.3 ± 0.0	4.3 ± 0.2
	fMLP	88.2 ± 0.1	2.5 ± 0.1	1.7 ± 0.3
	L-AA	78.8 ± 0.8	0.8 ± 0.1	14.7 ± 0.9

a) IL-8 at 10 nmol/L; fMLP at 100 nmol/L; L-AA at 50 μmol.
b) % of total PBL ± SD (of triplicate).
PBL subpopulations were gated according to their forward and side light scattering. The percentage of PMN, monocytes and lymphocytes was quantified before and after chemotactic migration at the indicated conditions.

determinants were higher on PMN than on differentiated HL-60 cells (Table 1). Thus, in vitro differentiated HL-60 cells have comparable but not identical phenotypes as their PBL counterparts.
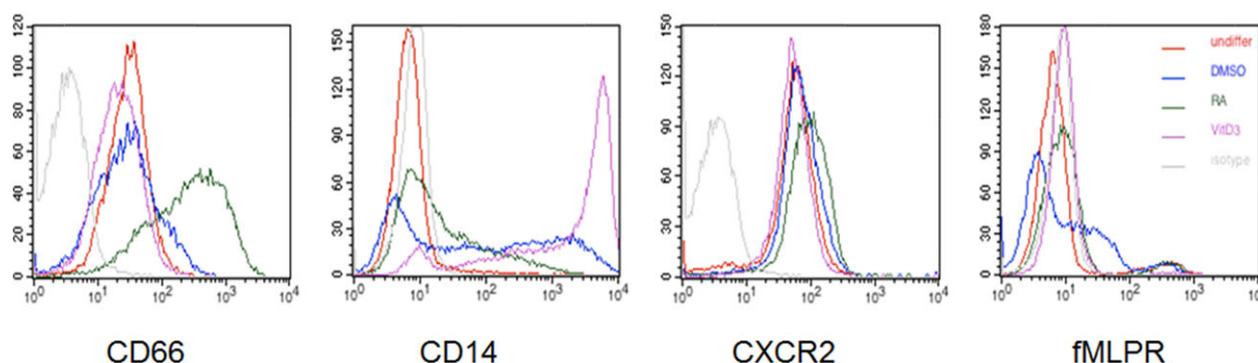
Figure 1. Expression of leukocyte surface antigens on undifferentiated and differentiated HL-60 cells. Histograms of cell surface fluorescence on undifferentiated HL-60 cells (red line), and HL-60 cells differentiated with DMSO (blue), vitD3 (violet), and RA (green) for 4 days. Staining with isotype-specific control antibodies are shown in gray line.

3.3 Chemokinesis and chemotaxis of differentiated HL-60

Cell movements in response to changes of the microenvironment is an important functional attribute of myeloid cells and neutrophils. Therefore, we analyzed the impact of L-AA on cell migration. Initially, CK was measured in HL-60 cells. In comparison to undifferentiated cells, dHL-60$_{DMSO}$ cells exhibited significant CK, which was also markedly increased in HL-60 cells differentiated by vit D3, RA, and L-AA (Fig. 3A). IL-8 or L-AA did not increase CK of dHL-60$_{DMSO}$ cells, while fMLP and GM-CSF even significantly mitigated it (Supporting Information Fig. 2). We also determined the impact of IL-8 and fMLP on CK of dHL-60$_{RA}$ and dHL-60$_{vitD3}$: their chemokinetic mobility was significantly less influenced by chemoattractants than in dHL-60$_{DMSO}$ cells (Supporting Information Fig. 2).

We investigated the CT in gradients of IL-8 and fMLP (Fig. 3B). dHL-60$_{DSMO}$ cells exhibited the strongest response to IL-8 and fMLP. dHL-60$_{DMSO}$ cells weakly migrated toward L-AA. dHL-60$_{vitD3}$ cells moved along fMLP gradients but distinctively less in IL-8 gradients. dHL-60$_{RA}$ cells similarly reacted to IL-8 and fMLP. L-AA was only chemotactic for dHL-60$_{DSMO}$ cells and dHL-60$_{vitD3}$ cells.

We analyzed the phenotypes of migrated cells. Cells that migrated against medium or L-AA had similar surface densities of CD14, CD66, fMLPR, and CXCR2 before and after migration (Fig. 4). This indicates that L-AA does not interact with receptors for chemo-attractants. Cells that migrated toward IL-8 had a blunted CXCR2 density. Accordingly, cells had less fMLPR after migration toward fMLP. Thus, receptor-mediated induction of CT downregulated the expression of the corresponding receptor.

We explored the relative contribution of L-AA to CT and CK of dHL-60$_{DMSO}$ cells in a checkerboard analysis, where the upper and lower compartments contain increasing amounts of the test substance [26] (Table 2A). While chemokinesis was increased by inclusion of 0–200 μmol/L L-AA (Table 2A, upper row), a gradient of L-AA did not further enhance cell mobility above the movement elicited by 50 μmol/L L-AA (Table 2A, central column). Physiological concentrations of L-AA had optimal effects on HL-60 cell mobility, while pharmacological concentrations rather impaired it.

3.4 Chemokinesis and chemotaxis of PBLs

Next, we investigated the effects of substances on PBL movement. Chemokinesis of PBLs was significantly increased by IL-8, fMLP, GM-CSF, and L-AA (Fig. 5A). The effects were most prominent for IL-8 and fMLP and less pronounced for GM-CSF and L-AA. This indicates that L-AA enhanced PBL movement. During chemotaxis, only PMNs responded to fMLP gradients (Supporting Information Fig. 3A). PBLs migrated toward fMLP and IL-8 in a concentration-dependent way (Supporting Information Fig. 3B); IL-8 and fMLP were the strongest chemo-attractants; GM-CSF was also a potent chemo-attractant and surpassed C5a significantly (Fig. 5B). PBLs migrated toward L-AA to a similar extent as toward C5a. L-AA triggered chemotaxis was larger at physiological (50 μmol/L) and reduced at pharmacological L-AA concentrations (1000 μmol/L). At those conditions, a low H_2O_2 production was only observed in medium containing 1000 μmol/L L-AA concentrations (Supporting Information Table 1).

We further determined the relative contribution of L-AA to CK and CT of PBL in a checkerboard analysis. CK was maximal when PBL were incubated with 50 μmol/L L-AA (upper rows in Table 2B). When cells were "loaded" with 200 μmol/L L-AA, cell mobility decreased (right column in Table 2B). Cells loaded with 50 μmol/L L-AA exhibited maximal CK and their migration was further enhanced in an L-AA gradient (middle column in Table 2B). Remarkably, high L-AA concentrations in the medium blunted CK. We infer from these data that
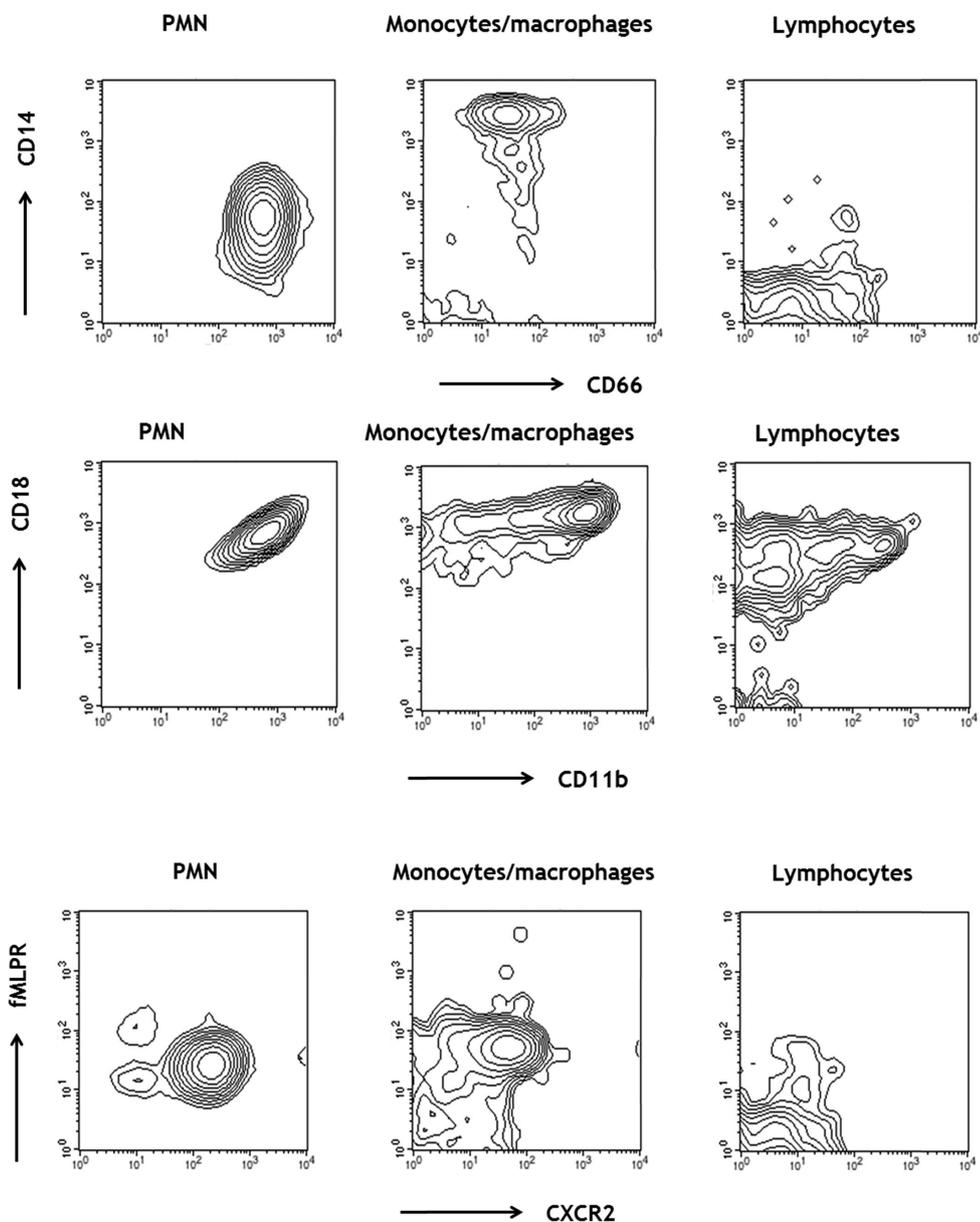
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Figure 2. Cytofluorometric analysis of peripheral blood leukocytes. Freshly isolated PBLs were stained with PE-conjugated anti-CD14 or anti-CD18 and APC-conjugated anti-CD11b or anti-CD66. PMN, monocytes/macrophages and lymphocytes were gated based on their characteristic forward (FSC) and side light scattering (SSC). The contour blots of each cell population were determined for the respective pair of PE- and APC-conjugated CD antibodies.

L-AA enhanced random migration and chemotaxis of PBL at concentrations similar to those observed in plasma.

3.5 Effects of chemo-attractants on the phenotypes of PBL

Light scatter and cytofluorometric analysis showed that PMN migrated in response to IL-8 and fMLP, while both PMN and lymphocytes responded to L-AA (Table 3). Transmigrated cells were virtually depleted of monocytes. PMN that migrated in IL-8 gradients expressed lower amounts of CXCR2, whereas migration elicited by fMLP resulted in downregulation of fMLPR (Fig. 6). Conversely, interaction with L-AA did not affected expression of CXCR2 and fMLPR. Cells that migrated at conditions of CK were phenotypically similar to those migrating at conditions of CT toward IL-8, fMLP, GM-CSF, or L-AA (not shown). Collectively, the data indicate that (a) neutrophils migrated toward chemo-attractants such as IL-8 and fMLP, but also in gradients formed by GM-CSF and
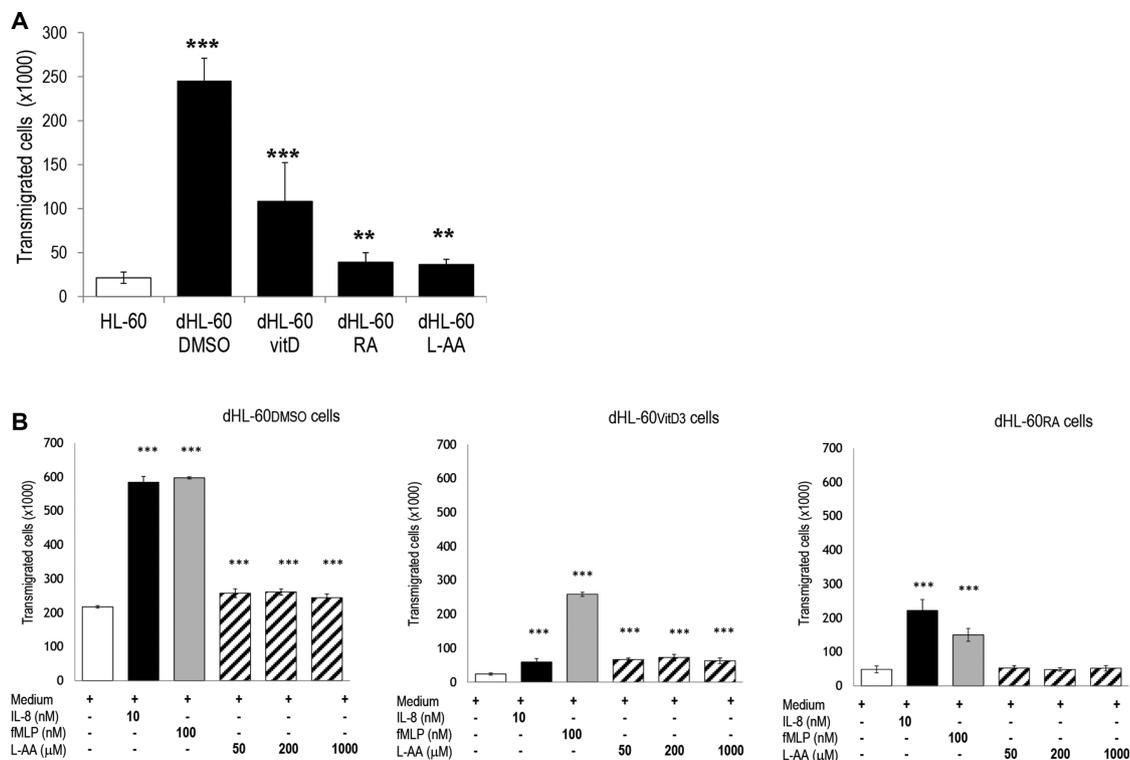
Figure 3. Chemokinesis and chemotaxis of dHL-60. CK and CT assays with undifferentiated and differentiated HL-60 cells were done as described in Materials and methods. Transmigrated cells were enumerated after 1 h. Results are shown as mean ± SEM (A) or mean ± SD of triplicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (versus undifferentiated HL-60 cells or medium control). (A) Chemokinesis of undifferentiated and 4 days differentiated HL-60 cells. Cells were placed in the upper compartment of Transwell plates containing CT medium. (B) Chemotaxis of dHL-60 $_{DMSO}$, dHL-60 $_{vitD3}$ and dHL-60$_{RA}$ cells. Cells were differentiated with DMSO, vitD3 or RA for 4 days, resuspended in chemotaxis medium and placed (2×10^6) in the insert Transwell plates. Upper and lower wells received medium, 10 nmol/L IL-8, 100 nmol/L fMLP, or 1 mmol/L L-AA.

L-AA, (b) these substances augmented migration capability above spontaneous movement.

3.6 L-AA altered the extent of chemotaxis in HL-60 cells and PBL

In order to test the hypothesis that L-AA modulated the chemotactic movement, we incubated HL-60 cells with L-AA before performing CT assays. L-AA was actively transported into HL-60 cells, since intracellular L-AA concentration exceeded that in the medium (Fig. 7A). Comparable to the observation made with PMN and PBMC [27], monocyte-like HL-60 cells (i.e. dHL-60$_{vitD3}$ cells) accumulated more cellular L-AA than their dHL-60$_{RA}$ counterparts. Cellular L-AA accumulation was dependent on the L-AA concentration in the medium and it was high during the critical period of the CT
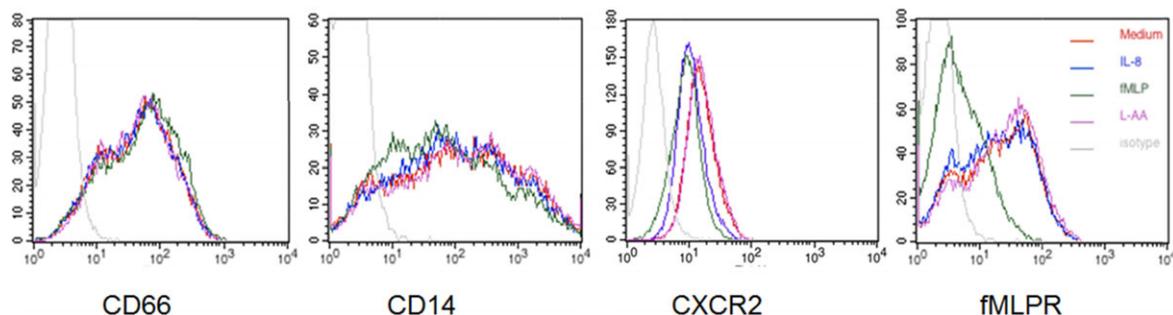

Figure 4. Phenotype of transmigrated HL-60 cells. Cytofluorometric analysis of dHL-60$_{DMSO}$ cells before and after CT towards IL-8 (10 nmol/L) (blue line), fMLP (100 nmol/L) (green), L-AA (1 mmol/L) (violet), or medium (red). Light gray line: isotype-matched control antibodies.
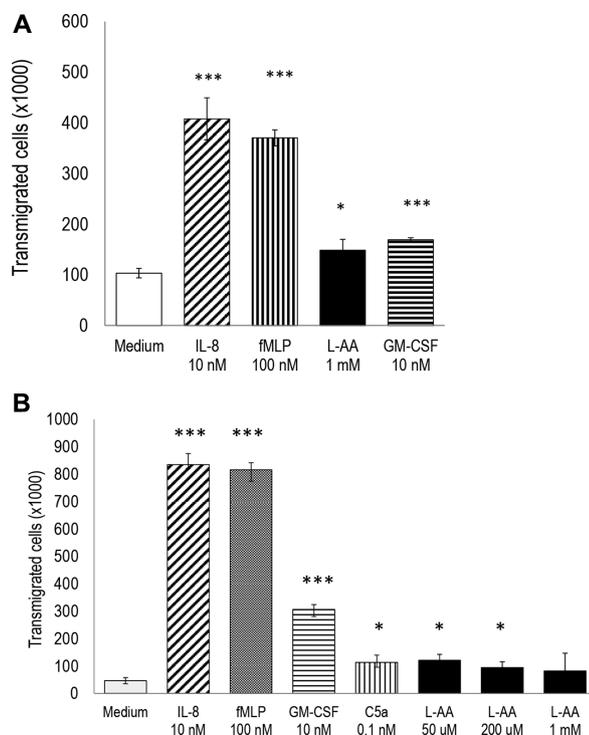
Figure 5. Chemokinesis and Chemotaxis of PBLs. CK and CT assays were performed as detailed in Materials and methods. Mean ± SD of triplicates. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ (versus medium control). (A) Chemokinesis of PBLs. PBLs (2×10^6 cells) were placed in the inserts of Transwell plates, where both compartments received medium, 10 nmol/L IL-8, 100 nmol/L fMLP, 1 mmol/L L-AA, or 10 nmol/L GM-CSF. Transmigrants were counted after 1 h. Mean ± SD of triplicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (versus PBL in medium). (B) Chemotaxis of PBLs. Transmigration of PBLs toward IL-8 (10 nmol/L), fMLP (100 nmol/L), GM-CSF (10 nmol/L), C5a (0.1 nmol/L), and L-AA 50–1000 μmol/L). Mean ± SD of triplicates. * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$.

assay [28], since L-AA concentrations remained elevated even in cells incubated for 1h in L-AA free medium (Supporting Information Fig. 4). dHL-60$_{DMSO}$ cells loaded with L-AA migrated more extensively in IL-8 and L-AA gradients, as compared to that of unloaded cells (Fig. 7B). In addition, fMLP and L-AA exerted a synergistic effect on CT: the number of transmigrated dHL-60$_{DMSO}$ cells in a gradient concomitantly

formed by fMLP and L-AA significantly exceeded the sum of transmigrants toward fMLP or L-AA (Fig. 7C). L-AA mediated enhancement of CT was not observed with dHL-60$_{vitD3}$ cells and dHL-60$_{RA}$ cells, which responded less in CT assays than dHL-60$_{DMSO}$ cells (Fig. 3B).

In order to study the effect of L-AA on CT of PBL, we first determined L-AA concentrations in PBLs from healthy individuals. The average L-AA concentration in freshly isolated PBLs was 1.02 ± 0.62 mmol/L (range 0.46–2.47 mmol/L, $N = 7$) (Supporting Information Fig. 5A, [27]). Incubating PBL's in L-AA containing medium marginally increased cellular L-AA, which plateaued at ∼2 mmol/L (Supporting Information Fig. 5B). Cellular L-AA level was maintained for at least 2 h, since it only moderately diminished during cell incubation in L-AA free medium (Supporting Information Fig. 5C). Next, freshly isolated PBLs were "loaded" with L-AA and used in chemotactic assays. At these conditions, CT toward fMLP was significantly enhanced (Fig. 8A). L-AA also influenced CT triggered by IL-8, since it synergistically augmented the CT towards the combined chemo-attractants (Fig. 8B). The impact of intracellular versus extracellular L-AA on CT was investigated by loading PBLs with L-AA or/and placing PBLs in Transwell compartments containing L-AA (Fig. 8C). While intracellular or/and extracellular L-AA only marginally influenced IL-8 dependent CT, intracellular L-AA significantly enhanced fMLP-dependent CT. Extracellular L-AA did not further increase CT boosted by intracellular L-AA. The weak chemotactic response to L-AA was not altered when PBL were loaded with L-AA, but it was significantly increased by extracellular L-AA and the combination of extracellular and intracelluar L-AA.

4 Discussion

Neutrophils or PMN have distinct cellular functions that are crucial for the homeostasis of the immune system. In the present study, we investigated the impact of retinoic acid (i.e. a vitamin A metabolite), vitamin D3 and L-AA on the functions of HL-60 cells and compared them with properties of PMN. Differentiation of HL-60 cells was induced with DMSO, vitamin D3 or retinoic acid as also described before [24,29,30]. dHL-60 cells acquired functional characteristics of PMN including chemotaxis towards fMLP [31], IL-8 [24], C5a [13],
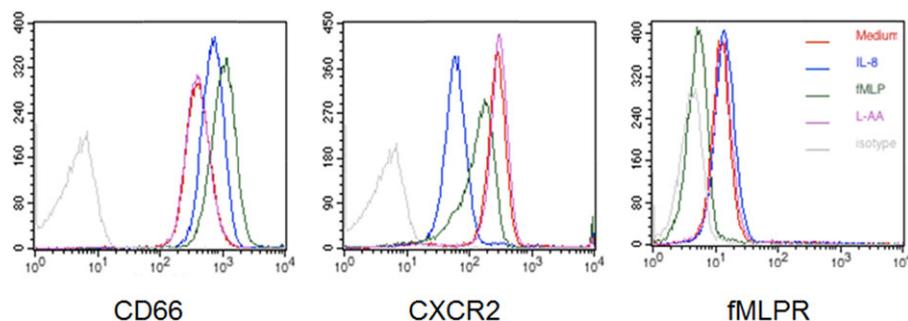

Figure 6. Phenotype of migrated PBLs. PBLs were incubated in Transwell plates and allowed to migrate in gradients of IL-8 (10 nmol/L) (blue line), fMLP (100 nmol/L) (green), or L-AA (50 μmol/L) (violet). Transmigrated cells were stained with antibodies against surface determinants and the fluorescence histograms overlaid with those before chemotaxis (red line). Light gray line: isotype-matched control antibodies.
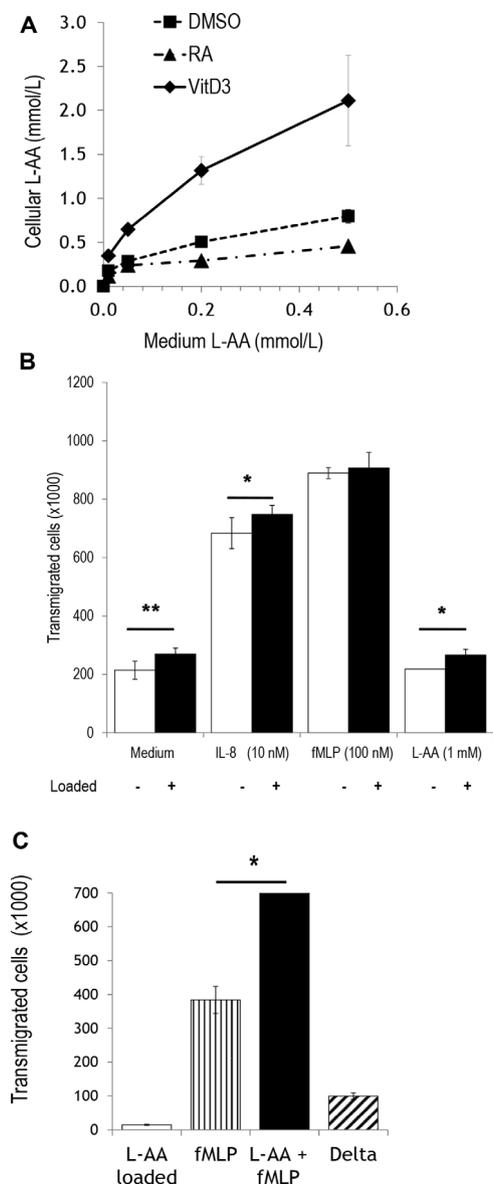
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Figure 7. L-AA influences chemotaxis of HL-60 cells. (A) L-AA is accumulated in HL-60 cells in relation to their differentiation status. In vitro differentiated HL-60 cells were incubated in PBS containing L-AA (0–0.5 mmol/L) for 1 h, washed and the cellular contents of L-AA determined. (B) Effect of L-AA on CT. HL-60$_{DMSO}$ cells were incubated with or without 50 μmoL/L L-AA for 1h and placed (2 × 10^6) in the Transwell inserts, which received L-AA (loaded +) or no L-AA (loaded –). Lower compartments received medium, 10 nmol/L IL-8, 100 nmol/L fMLP, or 1 mmol/L L-AA. Transmigrants were counted after 1 h. Mean ± SD of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (versus undifferentiated HL-60 cells). (C) Synergistic effects of L-AA and fMLP. dHL-60$_{DMSO}$ cells were resuspended in chemotaxis medium and placed (2 × 10^6) in the Transwell inserts. Lower wells received medium, L-AA (50 μmol/L), fMLP (100 nmol/L), or both. Transmigrated cells were counted after 1 h. Mean ± SD of triplicates. Medium control values were subtracted. Delta indicates the excess of transmigrated cells in the fMLP/L-AA combination versus the sum of transmigrated cells induced by each substance.
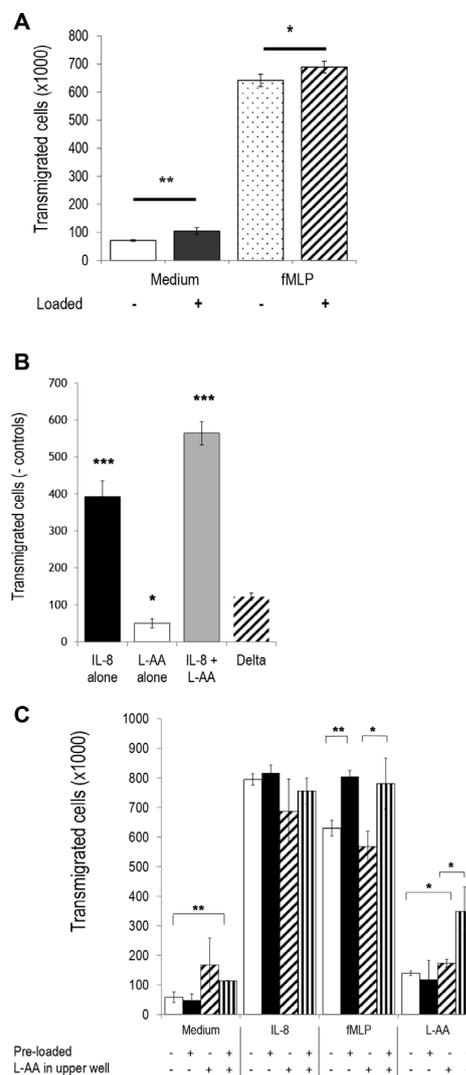
Figure 8. Influence of L-AA on chemotaxis of PBLs. (A) PBLs were incubated without or with 50 μmoL/L L-AA for 1 h, resuspended in chemotaxis medium and placed (2 × 10^6) in Transwell inserts, which received L-AA (loaded +) or not (loaded –). Lower compartments received medium or 1 nmol/L fMLP. Transmigrants were counted after 1 h. Mean ± SD of triplicates. (B) Synergistic effects of L-AA and IL-8. PBLs were resuspended in chemotaxis medium and placed (2 × 10^6) in the insert Transwell plates. Lower wells received medium, L-AA (50 μmol/L), IL-8 (10 nmol/L), or both. Transmigrants were counted after 1 h incubation. Mean ± SD of triplicates. Medium control values (2.08 ± 0.03 × 10^5 cells) were subtracted. Delta indicates the excess of transmigrated cells in the IL-8/L-AA combination versus the sum of transmigrated cells induced by each substance. Mean ± SD of triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (versus medium control). (C) Extracellular and intracellular L-AA influenced CT. PBLs were incubated with L-AA (50 μmol/L) (preloaded +) or without L-AA (preloaded –) for 1 h, resuspended in CT medium and placed in the inserts of Transwell plates. The upper compartment received 50 μmol/L L-AA where indicated. Cells transmigrating towards medium, IL-8 (10 nmol/L), fMLP (100 nmol/L), or L-AA (50 μmol/L) were measured after 1 h. Mean ± SD of triplicates. Significant differences (* $p < 0.05$, ** $p < 0.01$) due to the inclusion of L-AA are indicated.

and GM-CSF [28]. DMSO induced the expression of PMN markers CD11b, CD66c, CD14, and receptors for chemo-attractants. Phenotypically, dHL-60$_{DMSO}$ cells were of an inter-mediate neutrophil/monocyte cell type. Conversely, RA and vitamin D3 induced a neutrophil and monocyte phenotype, respectively (Fig. 1 and Supporting Information Fig. 1; [25,30, 32]). L-AA marginally affected the phenotype of HL-60 cells (Fig. 1). At pharmacological concentrations, L-AA induced the differentiation of HL-60 cells along the neutrophil pathway [33], but it also promoted apoptosis of cancer cells [27,34], pre-sumably due to L-AA dependent H_2O_2 production [35]. dHL-60$_{DMSO}$ cells exhibited the strongest chemotactic response to IL-8 and fMLP (Fig. 3B). Conversely, dHL-60$_{vitD3}$ cells and dHL-60$_{RA}$ cells mainly responded to fMLP and IL-8, respec-tively. These are functional hallmarks for monocytes and PMN [24]. L-AA markedly altered CK and CT of dHL-60$_{DMSO}$ cells and barely that of dHL-60$_{vitD3}$ cells and dHL-60$_{RA}$ cells, which also accumulated less cellular L-AA than dHL-60$_{DMSO}$ cells (Fig. 7A). Consequently, L-AA had a minor modulatory effects on CT of dHL-60$_{vitD3}$ cells and dHL-60$_{RA}$ cells. Since RA induced neutrophil-like HL-60 cells, which respond to in-termediary (IL-8) and end-target chemo-attractants (fMLP), it may enhance the innate immune response. Likewise, vitamin D3 promoted the monocyte/macrophage differentiation and thus the immune response against pathogens [36].

L-AA induced cell mobility of different PBLs including PMN and lymphocytes (Table 3). This was not mediated by receptors for chemo-attractants like fMLPR or CXCR2, since their expression was unaltered after L-AA induced CK and CT. Ligand-receptor interactions orchestrate cellular events that result in cell movement [2,37]. Given the fact that L-AA did not modify receptor expression and transiently upregulated sig-naling proteins [38], L-AA might trigger cell movement by re-arrangement of the cytoskeleton downstream of phosphatidyl inositol phosphate metabolites signaling pathways [15].

The implications of L-AA dependent enhancement of cell movement are numerous. PMN are short-living cells with key roles in defence against pathogens (via fMLPR) and in-nate immune responses induced by IL-8. PMN function is impaired by a compromised immune status of the host and declines during aging [39]. The data of this study suggest that PMN function is transiently improved by L-AA. The duration of this effect is limited by the very short half-life of PMN. Con-sequently, it is unlikely that chronic inflammation is favored by elevated systemic L-AA levels. Studies with neutrophils of CHS patients revealed that L-AA indeed enhanced neutrophil mobility [10], although pharmacological L-AA concentrations were required for in vitro "repair" of the defects [11]. Like-wise, millimolar concentrations of L-AA were required to al-ter granulocyte chemotaxis [13,40,41]. L-AA also improved PMN functions in neonates [17] and L-AA supplementation restored homeostasis of monocyte migration [42], whereas it had no effect on immune functions in ultramarathon runners [43]. Daily ingestion of 2–3 g of ascorbic acid enhanced neu-trophil chemotaxis [19]. In the present study, cell mobility was augmented at physiological L-AA concentrations, while CT

and CK was reduced at pharmacological L-AA concentration (Table 3). The overall contribution of L-AA to PBL movement was not assessed since PBLs from healthy individuals always contained substantial levels of L-AA (Supporting Information Fig. 5). In an animal model of scurvy, lymphocyte functions were impaired in the absence of L-AA [44]. Plausibly, L-AA influences apoptosis and thus the life-span of PMN [45–48].

L-AA is transported into neutrophils by a low affinity and a high affinity transporter (HAT), respectively [4,49,50]. HAT is critical when plasma L-AA concentration is <6 μmol/L, like in scurvy. The relationship between cellular L-AA contents and chemotactic functions is shown in this study. Since L-AA transport occurs within minutes [4,27], PMN rapidly respond to changing L-AA milieu [13]. This is in line with the obser-vation that both extracellular and cellular L-AA modulated the functional status of PMN (Fig. 8). The supplementation of individuals with different doses of L-AA will permit to measure its immediate effect on immune cells in vivo. We anticipate that the effects are measurable at ∼50 μmol/L of L-AA, which is readily reached by dietary means. Collectively, these data support the notion that L-AA contributes to the innate immune defence.

5   References

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