Lysophosphatidic acid inhibits the cytotoxic activity of NK cells: involvement of Gs protein-mediated signaling

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Abstract

Lysophosphatidic acid (LPA) is an activator and chemoattractant of NK cells, which are critical members of the immunological tumor surveillance machinery. Here, we analyzed the influence of LPA on the interaction of human NK cells with tumor cells such as the Burkitt lymphoma cell line Raji and the human melanoma cell line A2058. Thereby we found that LPA inhibits the release of perforin and cytotoxic activity of NK cells. Analysis of signal transduction showed that LPA induces common signaling pathways of chemotaxins such as Gi protein-dependent actin re-organization, activation of the mitogen-activated protein kinase p38 as well as phosphatidylinositol-3-kinase-dependent signal molecules [protein kinase B/Akt and glycogen synthase kinase-3β (GSK-3β)]. In contrast to most chemotaxins, LPA is also able to activate Gs-dependent signaling molecules. This signaling cascade involves the LPA receptor type-2, increase cAMP levels and protein kinase A (PKA) activation, which in turn are responsible for the modulatory effect of LPA on NK cell-mediated cytotoxicity. Moreover, blocking the regulatory subunits of PKA I abrogates the inhibitory effect of LPA, whereas the catalytic subunits are not involved. Based on our data, one can assume that LPA contributes to the tumor escape from the immunological surveillance machinery.

Introduction

NK cells are specialized leukocytes able to attack abnormal cells such as virus-infected cells or transformed tumor cells. In physiological conditions, NK cell cytotoxic activity is actively inhibited by receptors that recognize MHC class I molecules. Generally, normal cells express a threshold of MHC class I molecules that protect them from NK cell-mediated destruction, whereas reduced levels of MHC class I molecules in virus-infected or malignant cells render sensitivity to NK cell-mediated attack (1–3). The potential role of NK cells for cancer immunotherapy is linked to their capacity to recognize tumor cells without the requirement of pre-activation. Therefore, they might successfully attack tumors evading T cell killing by altered expression of HLA molecules (4). NK cells lyse target cells by release of cytoplasmic granules containing membrane pore-forming perforin and apoptosis-inducing DNA breakdown-mediated serine protease granzymes (5–7).

However, activation of NK cells is not solely regulated by reduction of MHC class I in conjunction with recognition of constitutive ligands. NK-activating receptors such as NK group (NKG)2D also participate in the regulation of NK cells (8). These receptors activate via adapter proteins phosphatidylinositol trisphosphate (PIP3)-generating type IA phosphatidylinositol (PI) 3-kinases or the Syk-family protein tyrosine kinases (1). Moreover, enhanced cytotoxic activity after stimulation with chemotaxins for NK cell such as macrophage inflammatory protein-1β (MIP-1β/CCL1), thymus- and activation-regulated chemokine (TARC/CCL17), regulate upon activation normal T cell expressed and secreted (RANTES/CCL5) and monocyte chemoattractant protein 1 (MCP-1/CCL-2) is well known, implicating a regulatory role in the functional balance between cytotoxicity inhibitory and activating receptors (9, 10). The above
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These reported effects were inhibited by pertussis toxin, implying coupling of LPA receptors to G proteins in NK cells (11). In addition, LPA stimulated chemotaxis, intracellular calcium mobilization and increased IFN-γ in NK cells. All these reported effects were inhibited by pertussis toxin, implicating coupling of LPA receptors to G proteins in NK cells (11). Moreover, these later findings might indicate creation of a favorable micromilieu by LPA in order to create immunological or/cytotoxic responses against tumors. However, histological investigations and clinical studies have shown that primary melanoma and metastasis can actually co-exist with effector cells of the immune system (e.g., dendritic cells, cytotoxic T cells and NK cells) (12). In the last years, several investigations pointed out that many functional aspects of the melanoma cells and their microenvironment contribute to tumor progression; e.g., the relevance of autocrine-secreted products such as CXCL1 and LPA in the growth response and metastatic process of melanoma cells has been demonstrated (13–15).

In order to better understand the role of LPA in the cross-talk between NK cells and tumor cells, we further characterized the influence of LPA in this context. Surprisingly, we found that LPA via LPA receptor type-2 enhanced intracellular cAMP, activated protein kinase A (PKA) and inhibited NK cell cytotoxicity.

Methods

Culture medium and reagents

rHu IL-2 (Proleukine) was from Chiron (Chiron GmbH, Ratingen, Germany); LPA (L-α-LPA, Oleoyl), myristoylated PKI14-22 cholera toxin, pertussis toxin and wortmannin were purchased from Sigma–Aldrich (Taufkirchen, Germany); 14:0 LPA was from Avanti Polar Lipids (Alabaster, AL, USA); mouse anti-CD3 and mouse anti-CD4 antibodies were purchased from Immunotools (Friesoythe, Germany); anti-human phospho-Akt (Akt)/protein kinase B (Ser473), Akt and phospho-glycogen synthase kinase-3β (pGSK-3β) as well as phospho-mitogen-activated protein kinase (MAPK) pp38 (Thr180/Tyr182) (p38) were purchased from Cell Signaling Technology (New England Biolabs GmbH, Frankfurt am Main, Germany); anti-actin was purchased from Santa Cruz Biotechnology (Heidelberg, Germany); anti-GSK-3β was from BD Transduction Laboratories (Heidelberg, Germany); Rp-8-Br-cAMPS was from Axxora Platform (Lorrach, Germany); NK culture medium consisted of RPMI 1640 supplemented with 10% human AB serum, 10 U ml⁻¹ penicillin, 10 U ml⁻¹ streptomycin and 1 mM L-glutamine (Promocell).

Preparation of IL-2-activated NK cells

The use of human cells was approved by the Research Ethics Board of the University of Jena. For each single experiment, IL-2-activated NK cells were isolated from blood of volunteers. Cells were prepared by adherence to plastic flasks of nylon wool column non-adherent cells in the presence of 1000 IU ml⁻¹ rHu IL-2. After 24 h, non-adherent cells were removed. Cells were grown for an additional 7–10 days as described by Inngjerdingen et al. (16). Thereafter, the cell suspension was depleted of T cells by binding to mouse anti-human CD3 and anti-human CD4 using anti mouse M-450 Dynabeads (Dynal, Oslo, Norway). This procedure resulted in a purity >95% of NK cell subsets expressing CD66.

Analysis of filamentous actin content

The intracellular filamentous actin content was analyzed by flow cytometry with Nitrobenzooxadiazol (NBD)-phallacidin staining. Briefly, aliquots of stimulated cell suspension (10⁶ ml⁻¹) were withdrawn at the indicated time intervals from samples incubated at 37°C. Equal volumes of cells (50 μl) were fixed in a 7.4% formaldehyde buffer and mixed with the staining mixture containing 7.4% formaldehyde, 0.33 μM NBD-phallacidin (MoBiTec, Göttingen, Germany) and 1 mg ml⁻¹ lysophosphatidylcholine (Sigma–Aldrich). The fluorescence intensity was measured by flow cytometry.

In vitro cytotoxicity assay

Cytotoxicity was determined with a standard ⁵¹Cr release assay. Target cells were labeled at 37°C for 1 h with 100 μCi Na₂⁵¹CrO₄ (Amersham, Freiburg, Germany). Cells were washed and re-suspended at cell density of 1 × 10⁶ cells ml⁻¹ with RPMI 1640 2% FCS medium. Effector and target cells in different ratios (10:1; 5:1 and 2.5:1) were placed into individual wells of 96-well U-bottom plates in a total volume of 200 μl at 37°C for 4 h. After incubation, 100 μl from the supernatants were mixed with MicroScint-40 cocktail (PerkinElmer, Jügesheim, Germany) and analyzed with a gamma counter (TopcountTM, Packard Instruments). The maximum or spontaneous release was defined as counts from samples incubated with 2% Triton-X or medium alone, respectively. The percentage of specific cytotoxicity was calculated as the measure of NK function against each target. Percentage specific lysis was calculated using the following formula:

\[
\frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100.
\]

Measurements of cAMP levels

Cells (1 × 10⁶ ml⁻¹) were stimulated with LPA (0.02–2 μM) and after the indicated time points lysed by addition of 100 μl lysis buffer. The amount of intracellular cAMP in the NK cell were maintained at 37°C in a 5% CO₂ incubator in RPMI 1640 supplemented with 10% fetal bovine serum, 10 U ml⁻¹ penicillin, 10 U ml⁻¹ streptomycin and 1 mM L-glutamine (Promocell).

Culture medium and reagents

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PKA activity assay

PKA activity in NK cell lysates was tested using StressXpress non-radioactive PKA activity kit (Biomol, Hamburg, Germany). This assay uses a specific synthetic peptide as a substrate for PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate. Briefly, NK cells (5 × 10^6 per sample) were treated with LPA for the indicated time points. Thereafter, cells were lysed in lysis buffer, centrifuged at 10 000 r.p.m. for 15 min and supernatants were frozen at ~70°C. A microtiter plate was soaked with 50 µl of kinase assay dilution buffer. After a washing step, 30 µl (corresponding to 10 µg of whole-cell lysate) of each sample was added. The reaction was initiated by addition of 10 µl ATP. The plate was incubated for 90 min at 30°C and phospho-specific substrate antibody was added. The level of antibody binding was estimated using secondary anti-rabbit IgG–HRP conjugate and corresponding substrate. Absorbance was determined using a microplate reader set at a wavelength of 450 nm. Kinase activity in cell lysates was calculated as follows:

\[
\text{Mean absorbance (sample) - Mean absorbance (negative control)}
\]

\[
\text{protein in cell lysate}
\]

Immunoblot analysis

Immunoblotting was performed by running the samples on SDS–PAGE gels (20 µg protein per lane) and transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 1 h at room temperature and then incubated with the first antibody (1:2000) overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibody (1:10 000) for 1 h at room temperature. Proteins were detected by ECL (Amersham).

Determination of perforin release

Perforin release in supernatant was measured using the Perforin ELISA kit (Diaclone, Besancon Cedex, France). Effector and target cells in different ratios (10:1, 5:1 and 2.5:1) were placed into individual wells of 96-well U-bottom plates in a total volume of 200 µl at 37°C for 4 h. After incubation, 150 µl from the supernatants were collected. Perforin levels were determined according to the manufacturer’s recommendations.

Expression of NK receptors

To stain NK cells with antibodies for various NK cell cytotoxicity receptors, these cells were incubated with either vehicle or 2 µM LPA for 4 h, washed and stained with 1 µg ml⁻¹ PE-conjugated mouse anti-human NK30 (CD337), 1 µg ml⁻¹ PE-conjugated mouse anti-human NKp44 (CD336), 1 µg ml⁻¹ PE-conjugated mouse anti-human NKG2D (CD314) or control PE-conjugated mouse IgG1 for 45 min at 4°C (antibodies were from Becton-Dickinson Pharmingen). The cells were washed twice and examined in the flow cytometer (FACSCalibur, Becton Dickinson Biosciences, San Jose, CA, USA).

Statistical analysis

Significant values (P < 0.05) were determined by using the two-tailed Student’s t-test.

Results

In order to better characterize the role of LPA on NK cells, intracellular signal transduction events and chemotaxis-associated intracellular re-arrangements such as actin polymerization were studied in cells obtained from different donors. Flow cytometric measurements indicated that LPA caused a rapid and concentration-dependent polymerization of actin molecules within 30 s (Fig. 1A). Detailed analyses showed ~70 to 80% higher mean fluorescence channels after stimulation with LPA for 30 s in comparison to control cells to go back to the initial values at ~300 s. Maximal effects were observed after stimulation with 2 µM LPA.

![Fig. 1. Effect of LPA on the actin polymerization in NK cells.](image)

(A) Cells were stimulated with LPA 20, 2 or 0.2 µM. The relative intracellular filamentous (F)-actin content was determined at the indicated time points by flow cytometry. At 30 and 60 s, the LPA stimulation showed significant (P = 0.04, 0.02 and 0.008 for 30 s and P = 0.02, 0.02 and 0.01 for 60 s) increase of the fluorescence when compared with the control. Data are means ± SEM (n = 3). (B) Cells were incubated without or with 100 ng ml⁻¹ pertussis toxin (Ptx) for 1 h at 37°C. Thereafter, cells were stimulated without or with 2 µM LPA and actin polymerization after 30 s of stimulation was analyzed. LPA significantly enhanced actin polymerization in NK cells at 30 s (P = 0.008), whereas the pre-treatment with Ptx significantly (P = 0.04) inhibited the LPA increase in the mean fluorescence. Data are means ± SEMs (n = 3). *P < 0.05, ns, not significant (P > 0.05).
To investigate the involvement of Gi proteins in this LPA response, NK cells were incubated with Gi protein-inactivating pertussis toxin. As shown in Fig. 1(B), pertussis toxin pre-treatment completely inhibited LPA-induced actin response. As a control to exclude toxicity induced by pre-treatment with the toxin, cells were stimulated with ionomycin and Ca\(^{2+}\) transients were monitored in Fura-2-loaded cells. Pertussis toxin did not influence Ca\(^{2+}\) transients triggered by this agent (data not shown).

Chemotaxins such as CCL5/RANTES stimulate PI 3-kinase and MAPK in leukocytes (17, 18). In the next set of experiments, activation and/or phosphorylation of different signal proteins downstream of PI 3-kinase were analyzed by western blot in NK cells obtained from different donors. These cells were treated with 2 \(\mu\)M LPA for the indicated time points (Fig. 2A). Probes were analyzed with antibodies against pAkt/protein kinase B (Ser473) and phospho-glycogen synthase kinase-3\(\beta\) (Ser9) (pGSK-3\(\beta\)) as well as phospho-MAPK pp38 (Thr180/Tyr182) (p38). The membranes were stripped and reprobed with anti-actin, anti-Akt (data not shown) and anti-GSK-3\(\beta\) (data not shown) to ascertain comparable amounts of the analyzed proteins in all samples. Basal levels of pAkt, pGSK-3\(\beta\) and pp38 activation were observed in LPA-unstimulated NK cells. Exposure of NK cells to LPA induced a further transient enhancement of pAkt, p-GSK-3\(\beta\) and pp38. Quantification of the luminescence indicated that LPA induced maximal levels of pAkt and pGSK-3\(\beta\) within 30 s with an increase by \(~60\) or 35\%, respectively. The kinetic of the pp38 response was slower peaking after 90 s with an increase of \(~50\%\) (data not shown).

**Fig. 2.** LPA-signaling pathway in NK cells. (A) NK cells were stimulated at 37°C with 2 \(\mu\)M LPA for 30, 60, 90, 120 and 180 s. After lysis, the samples were analyzed for pAkt, pGSK3\(\beta\), pp38 as well as actin by western blot. LPA induced maximal levels of pAkt and pGSK-3\(\beta\) within 30 s \((P = 0.0003\) for pAkt and 0.002 for pGSK-3\(\beta\)). The kinetic of the pp38 response peaked more slowly at 90 s \((P = 0.02\) Data from one representative experiment are shown. Experiment was repeated three times with NK cells isolated from different donors. (B–D) NK cells were incubated with or without 100 ng ml\(^{-1}\) pertussis toxin (Ptx) for 1 h at 37°C. Thereafter, cells were stimulated with or without 2 \(\mu\)M LPA. The effect of Ptx on LPA-induced responses to pAkt and pGSK-3\(\beta\) at 30 s and pp38 at 90 s was analyzed. Pre-treatment with Ptx significantly inhibited the LPA-induced enhancement of pAkt and pGSK-3\(\beta\) and pp38 \((P = 0.0003\) and pGSK-3\(\beta\) \((P < 0.0001\) responses. In contrast, Wtm neither influenced pp38 levels in unstimulated NK cells nor inhibited LPA-induced pp38 phosphorylation. Data are means of three experiments from different donors ± SEM. (E–G) The effect of wortmannin (Wtm) (100 \(\mu\)M) on LPA-induced responses on (B) pAkt at 30 s, (C) pGSK3\(\beta\) at 30 s and (D) pp38 at 90 s was analyzed. Wtm reduced the levels of pAkt and pGSK-3\(\beta\) in unstimulated NK cells and inhibited LPA-induced pAkt \((P = 0.0003\) and pGSK-3\(\beta\) \((P < 0.0001\) responses. In contrast, Wtm neither influenced pp38 levels in unstimulated NK cells nor inhibited LPA-induced pp38 phosphorylation. Data are means of three experiments from different donors ± SEM.
pre-treatment of NK cells with pertussis toxin did not influence the levels of pAkt, pGSK-3β and pp38 in unstimulated NK cells, but significantly inhibited the LPA-induced enhancement (Fig. 2B–D). In addition, short incubation of NK cells with the PI 3-kinase inhibitor wortmannin reduced the levels of pAkt (Fig. 2E) and pGSK-3β (Fig. 2F) in LPA-unstimulated NK cells and inhibited LPA-induced pAkt and pGSK-3β responses. In contrast, wortmannin did not influence pp38 levels in unstimulated NK cells and neither inhibited LPA-induced pp38 phosphorylation (Fig. 2G).

PI 3-kinase and p38 play pivotal roles in the regulation of the cytotoxicity in NK cells (19, 20). Moreover, an enhanced cytotoxic response has been described after NK cells exposure to chemotaxins such as CCL5/RANTES (10). Hence, we analyzed the effects of CCL5/RANTES and LPA on the cytotoxic activity of NK cells against the human Burkitt’s lymphoma cell line Raji. In accordance with the literature, CCL5/RANTES increased in a concentration-dependent manner NK cytotoxic activity by ~40% (Fig. 3A). In contrast to CCL5/RANTES, LPA inhibited the cytotoxic activity of NK cells against Raji by ~35 to 40% (Fig. 3B). To exclude the possibility that the interaction of LPA with the Raji target cell might cause a cell type-specific inhibitory effect on cytotoxicity, a second set of experiments with A2058 human melanoma cell lines was performed. Indeed, LPA significantly inhibited in a concentration-dependent manner the cytotoxic activity of NK cells against A2058 (Fig. 3C). In addition, similar inhibitory effects on NK cell cytotoxicity have been also observed at various effector:target (E:T) ratios (10:1 and 5:1) and with the HS294T and SK-Mel23 human melanoma cell lines (data not shown). These findings would suggest that chemotaxins differentially influence the cytotoxic activity of NK cells.

Moreover, NK cells secrete IFN-γ without any stimulation. However, we found an increased IFN-γ production by LPA-treated NK cells (data not shown) as was described by Jin et al. (11).

Chemotaxin receptors in leukocytes usually mediate their action by interaction with G protein-coupled receptors causing Ca²⁺ transients, actin reorganization and PI 3-kinase activation (11, 17). In contrast to the G protein-coupled CCL5/RANTES receptor signal, coupling of the different types of LPA receptors is pleiotropic including coupling to Gα proteins, which activate adenyl cyclase. Next, the influence of LPA on cAMP levels was analyzed in NK cells. After 4 min, stimulation of cells with LPA a significant and concentration-dependent increase of cAMP was observed (Fig. 4A). In addition, pre-treatment of NK cells with Gα protein-activating cholera toxin (0.5 μg ml⁻¹) caused an enhancement of intracellular cAMP levels in LPA-unstimulated NK cells and exposure of NK cells to LPA further increased the cAMP levels. In contrast, pre-treatment of NK cells with pertussis toxin neither influenced the cAMP levels in LPA-unstimulated NK cells nor inhibited the LPA-mediated increase in the cAMP response (Fig. 4B). Parallel cytotoxicity experiment after pre-treatment with cholera toxin and pertussis toxin was performed. As already described, cholera toxin pre-treatment inhibited the cytotoxic activity of LPA-unstimulated NK cells. The inhibitory effect of cholera toxin was further enhanced by LPA. In contrast, pertussis toxin did not influence the cytotoxic activity in either LPA-unstimulated or LPA-treated NK cells (Fig. 4C).

Classically, cAMP signaling leads to the immediate activation of PKA, resulting in release of two catalytic subunits, which are able to phosphorylate serine and threonine residues on specific substrate proteins, and two regulatory subunits, which are the primary receptors for cAMP. Although
both PKA type I and type II isoenzymes are present in leukocytes, there are reports indicating that the specific activation of PKA type I is sufficient for cAMP-mediated inhibition of T and B cell proliferation (21, 22), as well as NK cell cytotoxicity (23–25). Therefore, PKA activity in NK cells was analyzed. Fig. 5(A) shows that LPA treatment increased PKA activity in NK cells. In order to identify the subunit of PKA modulating cytotoxic activity, experiments were performed with different PKA inhibitors. Myristoylated peptide PKI14–22 (PKI peptide) is a specific blocker of the catalytic activity of PKA, whereas Rp-8-Br-cAMPS binds to the regulatory subunits I (RI) and prevents the PKA I holoenzyme dissociation as well as release of the regulatory subunits I (26). If the inhibitory effect of LPA is mediated via the PKA pathway, it is then expected that inhibition of PKA activity blocks the inhibitory effects of LPA. To test this prediction, we analyzed the ability of the PKA inhibitor PKI peptide to abrogate the inhibitory effect of LPA on the cytotoxic activity of NK cells. Using PKA activity kit (Stressgen Bioreagents), we found that pre-treatment with PKI peptide neither affected the cytotoxic activity of unstimulated NK cells nor influenced the LPA-induced inhibition (Fig. 5B). PKI peptide at 1 μM inhibited the PKA activity in cell lysates (Fig. 5C), indicating that this compound can get in human NK cells and inhibit PKA catalytic activity. These results indicate that the inhibitory effect of LPA on NK cell cytotoxicity is not mediated via the catalytic subunits of PKA. In contrast, pre-treatment of NK cells with Rp-8-Br-cAMPS, a blocker of the regulatory subunits, completely prevented the inhibitory effect of LPA (Fig. 5D). These findings may indicate that the RI subunits have signaling functions per se and that they mediate the inhibitory effect of LPA on NK cell cytotoxicity.

Expression of different type of LPA receptors especially LPA1, LPA2 and LPA3 has been reported in NK cells. To identify the LPA receptor subtype modulating cytotoxicity, experiments with subtype-specific receptor antagonist and agonist were performed. Dioctylglycerol pyrophosphate (DGPP) is an antagonist for LPA1 and LPA3 receptors (27, 28), whereas 14:0 LPA has a selective ability to activate the LPA2 (29). Pre-treatment of NK cells with Rp-8-Br-cAMPS, a blocker of the regulatory subunits, completely prevented the inhibitory effect of LPA (Fig. 5D). These findings may indicate that the RI subunits have signaling functions per se and that they mediate the inhibitory effect of LPA on NK cell cytotoxicity.

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receptors (27, 28). Pre-treating NK cells with DGPP did not affect the LPA-induced inhibition of NK cell activity. If the LPA2 receptor is indeed physiologically more critical than LPA1 and LPA3 for inhibiting the cytotoxicity of NK cells, challenging cells with the LPA2-selective agonist 14:0 LPA might lead to the inhibition of NK cell cytotoxicity. To examine this hypothesis, we compared the ability of LPA2-selective agonist 14:0 and the pan-receptor agonist 18:1 LPA to inhibit cytotoxic activity in NK cells. The 14:0 LPA-stimulated NK cells reduced the cytotoxic activity of NK cells similarly to the LPA and 18:1 LPA (Fig. 6B). Parallel cAMP measurements showed that DGPP does not influence the LPA-induced increase of the cAMP levels. The 14:0 LPA agonist significantly enhanced cAMP levels as well as the pan-receptor agonist 18:1 LPA (Fig. 6C). Although no significant differences were found, stimulation with the LPA2-selective agonist 14:0 LPA was more effective than with the LPA and 18:1 LPA in increasing cAMP levels and inhibiting the cytotoxicity of NK cells; this finding implicates involvement of the LPA2 receptor. Taken together, the data presented suggest that LPA receptor subtype-2 rather than LPA receptor subtypes-1 and -3 may play a critical role in activating adenylcyclase and modulating the cytotoxicity by LPA in NK cells.

NK cells mediate their function through different receptors involving the release of cytotoxic granules containing perforin. Hence, to better characterize the effect of the LPA on NK cell cytotoxicity, we analyzed the release of perforin in co-cultures of NK cells with A2058 melanoma cells. After 4 h incubation in the absence or presence of LPA, supernatant was collected and perforin levels were quantified by ELISA. Figure 7 shows that LPA significantly inhibited in a concentration-dependent manner the release of perforin of NK cells against A2058 melanoma cells. In addition, similar inhibitory effects on the perforin release have been also observed at various effector:target ratios and with the HS294T and SK-Mel23 human melanoma cell lines (data not shown).

Additionally, we analyzed the effect of LPA in the expression of NK cell receptors. NK cytotoxicity receptors NK30 (CD337), NKp44 (CD336) and NKG2D (CD314) were analyzed after 4-h stimulation with or without LPA. We found no difference between LPA versus control treatment in the expression of NK cytotoxicity receptors (Fig. 8). Similarly, we could not see the influence of LPA on the NK cell inhibitory receptors [killer cell Ig-like receptor (KIR)]; KIR2DL2 (CD158b1), KIR2DL3 (CD158b2) and KIR2DS2 (CD158j), KIR2DL1 (CD158a) and KIR2DS1 (CD158h) (data not shown).

Discussion

LPA was originally identified as a key molecule in de novo lipid biosynthesis. Meanwhile, it has been also regarded as an important extracellular mediator regulating a broad range of biological functions such as platelet aggregation, smooth muscle contraction, cell proliferation and immunological reactions (30, 31). It has been also identified as an activator of oxygen production in eosinophils and regulator of

Fig. 5. LPA-induced activation of PKA in NK cells. (A) NK cells were treated with 2 μM LPA for 1, 4, 15 and 30 min. PKA activity in cell lysates was tested using StressXpress non-radioactive PKA activity kit. Data are means ± SEM of three experiments with cells from different donors. After 4 min stimulation, LPA significantly (P = 0.026) increased PKA activity in comparison to controls. NK cells were preincubated for 30 min with the inhibitors before target cells and LPA were added. (B) Effect of PKI peptide (1 μM) on the lytic activity of NK cells. (C) Effect of PKI peptide (1 μM) on the PKA activity in cell lysates. (D) Effect of Rp-8-Br-cAMP (1 mM) on the lytic activity of NK cells. Pre-treatment with PKI peptide neither affected the cytotoxic activity of unstimulated NK cells nor influenced the LPA-induced inhibition. In contrast, pre-treating NK cells with Rp-8-Br-cAMPS abrogated the inhibitory effect of the LPA (P = 0.02). Data are means ± SEMs of three experiments with cells from different donors.
More-over, LPA is able to stimulate chemotaxis and secretion of IFN-\(\gamma\) in NK cells (11). It has been suggested that these later findings might create a favorable micromilieu for immune cells in order to create immunological or/and cytotoxic responses against tumor cells. LPA can be rapidly synthesized by different pathways involving diacylglycerol activation or sphingomyelinase conditioning of cell-derived plasma membrane vesicles followed by a secretory type II phospholipase A2-dependent metabolism (34, 35). Additionally, specific oxidative degradation in minimal-oxidized low-density lipoproteins results in production of LPA (36). Moreover, lysophospholipase D is able to catalyze the production of LPA from lysophosphatidylcholine. It is currently assumed that autocrine or paracrine mechanism involving lysophospholipase D-generated LPA might contribute to tumor cell motility, survival, or proliferation of melanoma cells and prostate cancer (14). Therefore, current knowledge of biological function of LPA points to favorable as well as hostile actions on tumor cells. In order to better understand the role of LPA in the cross-talk between NK cells and tumor cells, we further characterized the influence of LPA in this context.

Recently, expression of LPA receptor type-1, -2 and -3 and \(G_i\) protein-mediated activity of LPA on chemotaxis and Ca\(^{2+}\) transients in NK cells has been shown (11). It has been suggested that these later findings might create a favorable micromilieu for immune cells in order to create immunological or/and cytotoxic responses against tumor cells. LPA can be rapidly synthesized by different pathways involving diacylglycerol activation or sphingomyelinase conditioning of cell-derived plasma membrane vesicles followed by a secretory type II phospholipase A2-dependent metabolism (34, 35). Additionally, specific oxidative degradation in minimal-oxidized low-density lipoproteins results in production of LPA (36). Moreover, lysophospholipase D is able to catalyze the production of LPA from lysophosphatidylcholine. It is currently assumed that autocrine or paracrine mechanism involving lysophospholipase D-generated LPA might contribute to tumor cell motility, survival, or proliferation of melanoma cells and prostate cancer (14). Therefore, current knowledge of biological function of LPA points to favorable as well as hostile actions on tumor cells. In order to better understand the role of LPA in the cross-talk between NK cells and tumor cells, we further characterized the influence of LPA in this context.

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proteins by ADP-ribosylation (37). This set of data is in accordance with signaling pathways induced by chemotaxins such as chemokines, complement fragment C5a of N-formylpeptides in leukocytes, which predominately activate Gi protein-coupled receptors.

PI-3 kinase and p38 play pivotal roles in the regulation of NK cell cytotoxicity (19, 20) and enhanced cytotoxicity of NK cells is described after stimulation with chemotaxins, such as RANTES/CCL5, MIP-1β/CCL1, TARC/CCL17 and MCP-1/CCL-2 (9, 10, 38). Surprisingly, we detected that CCL5/RANTES and LPA differentially influenced the NK cell cytotoxicity. In accordance with the literature, RANTES/CCL5 enhanced the cytotoxicity of NK cells toward tumor cells, whereas LPA inhibited the release of perforin and cytotoxic activity. Besides coupling to Gi protein, heterologous expression studies showed that LPA receptor type-1 is also able to interact with Gq and G12, LPA receptor type-2 with Gq, G12 and Ga, and LPA receptor type-3 with Gq (29, 30, 39). Indeed, LPA was able to enhance cAMP levels in NK cells and stimulated activation of cAMP-dependent PKA. These results indicate that LPA in contrast to classical chemokatins such as CC chemokines stimulates Gi as well Ga protein-dependent signaling pathways in NK cell.

NK-activating and inhibitory receptors interact with ligands on the target cells, and the integration of signals transmitted by these receptors determines the response. NK cell activity is repressed upon recognition of both classical and non-classical HLA class I molecules (40). In contrast, activating NK cell receptors trigger the release of cytolytic granules and induce cytokine production. Established activating receptors on NK cells include the activating KIRs, CD16 (low-affinity Fc receptors for IgG), the natural cytotoxicity receptors and the NKG2 (41). We did not find the influence of LPA in the receptor profile on NK cell surface but we have shown that the inhibitory effect of LPA in the cytotoxic activity of NK may have an influence in the levels of perforin released to the target cells.

Cholera toxin, which continuously activates adenylyl cyclase via ADP-ribosylation of Ga proteins, has been reported to inhibit the cytotoxic activity of NK cells (42). Moreover, reduced cytotoxic activity has been reported after exposure of NK cells to Ga protein-stimulating ligands like adenosine, prostaglandin E2 and prostaglandin D2 (43–45). Therefore, our data are consistent with the concept that cAMP regulates crucial steps in the cytotoxic activity of NK cells. Moreover, our results with the selective LPA receptor type-2 agonist 14:0 and the LPA receptor type-1 and -3 antagonist DGPP demonstrate that LPA receptor type-2, rather than LPA receptor type-1 and -3, plays a critical role in activation of adenylylcyclase and modulation of cytotoxicity by LPA. This signal transduction event fits well with heterologous expression studies reporting coupling of LPA receptor type-2 to Ga proteins. In addition, the idea of modulation of cytotoxicity in NK cells by Ga protein-dependent signal pathways is further corroborated here using PKA inhibitors. Currently, two different isoenzymes of the PKA are known (46) and that...
NK cells express both subtypes (23). Although the catalytic subunits of both PKA types are similar, the regulatory subunits may have distinctive functions. We found that direct inhibition of the catalytic subunit with PKI peptide does not abrogate the inhibitory effect of LPA but blocking the release of regulatory subunits by Rp-8-Br-cAMP attenuates LPA-mediated inhibition of the cytotoxicity. Similar effects were previously reported with adenosine and murine NK cells (24). These data indicate that the inhibitory effects of LPA in NK cell cytotoxicity is mediated via cAMP-mediated release of regulatory subunits from PKA I.

In this context, it is worth mentioning that functional activities have been attributed to the regulatory subunits of PKA. Mice with a selective knockout of genes encoding regulatory subunits show distinctive phenotypic changes (21, 46). Regulatory subunit RIIα−/− mice present early embryonic lethality, whereas regulatory subunit RIIα knockout mice show no detectable abnormality in PKA activity. Regulatory subunit RIIβ knockout mice have hippocampal alterations, reduce inflammatory responses, nociceptive pain and metabolic changes without changes in a total PKA activity. Regulatory subunit RIIβ−/− manifested metabolic changes, diminished motor learning, increased alcohol consumption and decreased alcohol-induced sedation (22, 47).

In summary, we demonstrated here that the LPA inhibits the cytotoxicity of NK cells enhancing cAMP levels and activating PKA I. Therefore, one can assume that activities of NK cells are impaired in vivo by local production of LPA. In the last years, several functions of LPA in respect to the growth response and metastatic process of melanoma cells have been demonstrated. Based on this data, one can also assume that LPA contributes to the escape of tumor cells from immunological surveillance machinery.

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Abbreviations
DGPP diocylglycerol pyrophosphate
KIR killer cell Ig-like receptor
GSK-3β glycogen synthase kinase-3β
LPA lysophosphatidic acid
MIP-1β macrophage inflammatory protein-1β
MCP-1 monocyte chemoattractant protein 1
NKG NK group
pAkt phospho-Akt
PAP phophatidylinositol
PAP3 phosphatidylinositol trisphosphate
PKA protein kinase A
RANTES regulate upon activation normal T cell expressed and secreted
TARC thymus- and activation-regulated chemokine

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