Brief Definitive Report

Triggering of Human Monocyte Activation through CD69, a Member of the Natural Killer Cell Gene Complex Family of Signal Transducing Receptors

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Summary
The expression and function of CD69, a member of the natural killer cell gene complex family of signal transducing receptors, was investigated on human monocytes. CD69 was found expressed on all peripheral blood monocytes, as a 28- and 32-kD disulfide-linked dimer. Molecular cross-linking of CD69 receptors induced extracellular Ca\(^{2+}\) influx, as revealed by flow cytometry. CD69 cross-linking resulted also in phospholipase A\(_2\) activation, as detected by in vivo arachidonic acid release measurement from intact cells and by direct in vitro measurement of enzymatic activity using radiolabeled phosphatidylcholine vesicles. Prostaglandin E\(_2\), 6-keto-prostaglandin F\(_1\alpha\), and leukotriene B\(_4\) were detected by radioimmunoassay in supernatants from CD69-stimulated monocytes, suggesting the activation of both cyclooxygenase and lipoxygenase pathways after CD69 stimulation. CD69 cross-linking, moreover, was able to induce strong nitric oxide (NO) production from monocytes, as detected by accumulation of NO oxidized derivatives, and cyclic GMP. It is important to note that NO generation was responsible for CD69-mediated increase in spontaneous cytotoxicity against L929 murine transformed fibroblast cell line and induction of redirected cytotoxicity towards P815 FcRII\(^+\) murine mastocytoma cell line. These data indicate that CD69 can act as a potent stimulatory molecule on the surface of human peripheral blood monocytes.

CD69 is a type II integral membrane protein with a C-type lectin binding domain. It is a member of the natural killer cell gene complex (NKC) family of cell surface receptors, as it is the product of a single gene located on synthetic regions of chromosome 12 in humans and chromosome 6 in the mouse, and displays extended sequence homology with the other members of the family (1-4). CD69 is induced on the surface of recently activated lymphocytes (5-9), likely through a mechanism requiring p21\(^{ras}\) activation (10). In vivo, CD69 is found constitutively expressed on activated lymphoid cells, including CD3\(^{bright}\) thymocytes, lymph node and tonsil T cells, and gut mucosal T lymphocytes (11-13). However, CD69 expression is not restricted to activated lymphocytes, as activated neutrophils and eosinophils can express CD69 (14, 15). Moreover, platelets, epidermal Langerhans cells, and bone marrow myeloid precursors express CD69 constitutively (16, 17). Such a wide distribution suggests a more general role for the CD69 receptor in the biology of hematopoietic cells, as supported also by the observation that in all cell types investigated, CD69 cross-linking generates an intracellular signal (for a review see reference 18).

We report here on the expression and function of CD69 on human circulating monocytes. Our data indicate that circulating monocytes express functional CD69 receptors on their cell surface and that the signal generated after CD69 cross-linking is a potent activator of monocyte functions, including the production of inflammation mediators and the induction of cytotoxic activity.

Materials and Methods

Isolation of Human Monocytes. Human PBMC were isolated from buffy coats by lymphoprep gradient centrifugation (Nycoderm Pharma AS, Oslo, Norway) and fractionated on a two-step (43 and 45.5%) Percoll gradient centrifugation. Monocytes obtained from the Percoll interface were usually 80-90% pure as assessed by anti-CD14 staining and flow cytometry analysis. Contaminating T, B, and NK cells were eliminated by negative immunomagnetic selection by using anti-CD2- and anti-CD19-coupled beads (Dynal, Wirral Merseyside, UK). This procedure routinely resulted in a...
>95% pure monocyte population, as measured by anti-CD14 staining and flow cytometry analysis.

**Antibodies, Immunofluorescence, and Flow Cytometry Analysis.**

Purified anti-CD69 (anti-Leu23, IgG1) and purified anti-CD45RA (anti-Leu18, IgGl) were from Becton Dickinson (San Jose, CA). Anti-CD69 F(ab')2 and control mouse F(ab')2 were prepared as previously described (16). F(ab')2 goat anti-mouse (GaM) was from Organon Teknika Corp. (Durham, NC). For surface staining, PBMC at 10^7/ml were incubated for 30 min at 4°C with appropriate amounts of the following directly conjugated antibodies (from Becton Dickinson): PE anti-CD14 (anti-LeuM3), FITC anti-CD69 (anti-Leu23), and isotype-matched FITC and PE control antibodies. Samples were analyzed by flow cytometry on a FACSScan® (Becton Dickinson).

**Labeling, Immunoprecipitation, and SDS-PAGE Analysis.**

3 x 10^7 purified monocytes were washed twice and resuspended in Dulbecco's PBS without Ca^{2+} and Mg^{2+}. 125I-labeling and lysis in 0.5 ml 0.5% NP-40 lysis buffer were then performed as previously described (16). Cell lysates were preclared three times with protein G-Sepharose (Sigma Chemical Co., St. Louis, MO) and then incubated for 2 h at 4°C with anti-CD69 bound to protein G-Sepharose. Immunoprecipitates were run on a 10% SDS-PAGE. Gels were fixed, dried, and autoradiographed.

**Ca^{2+} Flux Measurements.**

Purified monocytes at 10^7/ml were loaded with 1 μg/ml Fluoronic F 127 and 3 μm Fluo-3 (Molecular Probes, Inc., Eugene, OR) for 45 min at 37°C in RPMI-1640 with 1% FCS, which was employed during the whole procedure. Cells were then washed and incubated with 10 μg/ml anti-CD69 F(ab')2 or control mouse F(ab')2. After two other washes at room temperature, cells were kept 10 min at 37°C and immediately analyzed before and after stimulation with 10 μg/ml F(ab')2, GaM by flow cytometry with a FACSScan® as previously described (13).

**Arachidonic Acid Release.**

Monocytes were incubated in serum-free medium (RPMI-1640) for 4 h at 37°C with 1 μCi/ml [5, 6, 8, 9, 11, 12, 14, 15,3H]-arachidonic acid (AA) (Amersham, Bucks, UK). Labeled cells were then washed twice, resuspended in fresh medium containing 0.01% fatty acid-free BSA (Sigma Chemical Co.) and stimulated with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2 and 10 μg/ml F(ab')2 GaM. AA release assay was carried out in a shaking incubator bath at 37°C, and the reaction stopped by centrifugation at 14,000 rpm for 1 min. The 3H content of the supernatant was estimated in a β counter.

**Phospholipase A2 Activity Assay.**

The cells were treated with anti-CD69 mAb (10 μg/ml) or control anti-Leu18 mAb, and GaM (10 μg/ml) for 10 min at 37°C and lysed by sonication. Radiolabeled phosphatidylcholine (PC) vesicles were prepared by sonicating liposomes composed of 1 μCi/ml [3H]arachidonic acid, 200 μM CaCl_2, 5 mM MgCl_2, and 0.1% fatty acid-free BSA) with the radiolabeled phospholipid, r-a-dipalmitoyl [choline-methyl-3H] containing 10 μg/ml F(ab')2 GaM. After 18 h incubation, supernatant was removed from each well and counted. Assay of phospholipase A2 activity against fibroblast-transformed cell line L929 was performed by incubating monocytes with 5 x 10^3 51Cr-labeled (Na_2^{51}CrO_4; New England Nuclear) tumor cells in triplicate wells, in the presence of 10 μg/ml anti-CD69 or anti-CD45RA mAbs. After 18 h incubation, supernatant was removed from each well and counted. Assay of phospholipase A2 activity with the radiolabeled phospholipid, r-a-dipalmitoyl [choline-methyl-3H], was performed by incubating monocytes with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, and 10 μg/ml F(ab')2 GaM. After 18 h incubation, supernatant was removed from each well and counted. The 3H content of the supernatant was estimated in a β counter.

**Results and Discussion**

**Monocytes Constitutively Express CD69.**

To determine whether monocytes constitutively express CD69, purified monocytes were stained with anti-CD14 and FITC anti-CD69 mAb. Isotype-matched FITC and PE mouse Ig were used as negative controls. Samples were analyzed on a FACSScan® and data were presented in the form of a two-parameter contour plot. Fig. 1A shows that circulating monocytes, as identified by CD14 expression, constitutively express CD69 molecules.

**Measurement of Nitric Oxide Synthesis.**

Nitric oxide (NO) synthesis was measured as NO_2^- accumulation. In aqueous solution, NO reacts rapidly with O_2 and accumulates in the culture medium as NO_3^- and NO_3^- ions. Monocytes were incubated with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, together with 10 μg/ml F(ab')2 GaM, and 100 μl of the culture supernatants were removed for NO_2^- measurement at the indicated times. NO_2^- was quantified by a colorimetric assay based on the Griess reaction. The OD was measured at 550 nm using a micro-ELISA reader (Easv Reader EAR 400; Kontron Analytic, Milan, Italy).

**Cyclic GMP Assay.**

Monocytes were incubated with 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, together with 10 μg/ml F(ab')2 GaM. The reaction was stopped at the indicated times by addition of ice-cold TCA (10%, w/v, final concentration). The extraction and the assay were performed as directed in the RIA kit (New England Nuclear).

**Cytotoxicity Assay.**

Assay of monocyte-redireced cytotoxicity against murine mastocytoma cell line P815 was performed by incubating monocytes with 5 x 10^3 51Cr-labeled (Na_2^{51}CrO_4; New England Nuclear) tumor cells in triplicate wells, in the presence of 10 μg/ml anti-CD69 or anti-CD45RA mAbs. After 18 h incubation, supernatant was removed from each well and counted. Assay of cytotoxic activity against fibroblast-transformed cell line L929 was performed by incubating monocytes with 5 x 10^3 3H thymidine-labeled (New England Nuclear, Inc.) cells, in the presence of 10 μg/ml F(ab')2 anti-CD69 or control mouse F(ab')2, and 10 μg/ml F(ab')2 GaM. After 18 h incubation, supernatant was removed from each well and counted. The percentage of specific lysis was calculated as follows: 100 x [(experimental release - spontaneous release)/(maximum release - spontaneous release)]. NO synthase inhibitor, 1-μM-monomethyl arginine (1-NAME; Wellcome, Beckenham, Kent, UK), was used at 0.5 mM and had no effect on target cell viability or spontaneous [3H]thymidine or 35Cr release.

**CD69 Cross-linking Induces Ca^{2+} Influx and Phospholipase A2 Activation in Monocytes.**

CD69 cross-linking has been shown to induce [Ca^{2+}]_i elevation in lymphocytes, platelets, and granulocytes (14, 16, 20). To establish whether CD69 molecules were able to efficiently transduce a signal in monocytes, we initially measured intracellular [Ca^{2+}]_i levels after stimulation with F(ab')2; anti-CD69, together with GaM F(ab')2 to maximize antibody cross-linking. Fig. 2A shows...
that CD69 triggering in monocytes results in rapid and sustained [Ca\(^{2+}\)]\(_{i}\) elevation. This is most likely due to extracellular Ca\(^{2+}\) influx, since EGTA addition to the medium completely abolished CD69-induced intracellular [Ca\(^{2+}\)]\(_{i}\) elevations.

CD69 stimulation on platelets has been shown to result in selective phospholipase A2 (PLA2) activation, and to be sufficient to lead to platelet degranulation and aggregation (19). To determine whether monocyte CD69 activates PLA2, monocytes were labeled with \(^{3}H\)-AA and stimulated with anti-CD69 mAbs. Fig. 2B shows that \(^{3}H\)-AA-labeled monocytes released high amounts of radioactive species in the medium after CD69 stimulation. To directly measure PLA2 enzymatic activity, cell extracts from CD69-stimulated monocytes were incubated with vesicles containing radiolabeled PC. After the in vitro reaction, phospholipids were extracted and chromatographed on TLC plates, and lyso-PC was detected and quantitated. Fig. 2C shows that cell extracts from CD69-stimulated monocytes caused substantial release of lyso-PC from PC vesicles, demonstrating that CD69 is functionally associated to a PLA2 in monocytes. Taken together, these data indicate that CD69 receptors may operate a very similar signal-transducing apparatus, which includes extracellular Ca\(^{2+}\) influx and PLA2 activation, in different cell types.

**CD69 Cross-linking Induces Production of both Cyclooxygenase and Lipoxygenase Metabolites.** Most AA produced after PLA2 activation is oxidized through two main pathways, the lipoxygenase pathway, producing leukotrienes, and the cyclooxygenase pathway, giving rise to prostanoids. Both leukotrienes and prostanoids are important mediators of inflammation (21). We therefore investigated whether AA was undergoing metabolic oxidation in monocytes after CD69 triggering. Fig. 3 shows that CD69 cross-linking in monocytes induced both cyclooxygenase and lipoxygenase products, as indicated by the release in the medium of CD69-stimulated monocytes of amounts of PGE\(_2\alpha\), 6-keto-PGF\(_{1\alpha}\), and LTB\(_4\) comparable with those generated by LPS stimulation. Considering

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**Figure 1.** (A) Two-color immunofluorescence analysis of human PBL stained with anti-CD14 PE (y axis) and anti-CD69 FITC (x axis). PBL stained with control PE and FITC mAbs were contained within the lower left quadrant (data not shown). Four donors gave similar results. (B) SDS-PAGE analysis of anti-CD69 immunoprecipitates from human monocytes. Purified monocytes were \(^{125}I\)-labeled, then material immunoprecipitated with anti-CD69-bound protein G-Sepharose (lanes A and C), or pre cleared with protein G-Sepharose (lanes B and D), analyzed in SDS-PAGE under nonreducing (NR, lanes A and B) and reducing (R, lanes C and D) conditions.

**Figure 2.** Anti-CD69 induced Ca\(^{2+}\) signaling and PLA2 activation in human monocytes. (A) Purified monocytes were loaded with Fluo-3, labeled with 10 \(\mu\)g/ml anti-CD69 F(ab')\(_2\) (open triangles) or control mouse F(ab')\(_2\) (open circles) and analyzed by FACS\(^{®}\) before and after stimulation at time 0 with 10 \(\mu\)g/ml F(ab')\(_2\) GaM. 2,000 events were recorded every 20 s. Stimulation of CD69-labeled cells was also performed in the presence of 2.5 mM EGTA (closed triangles). Data from one representative donor out of six tested, giving similar results, are shown. (B) Monocytes were labeled with \(^{3}H\)-AA and stimulated with 10 \(\mu\)g/ml anti-CD69 F(ab')\(_2\) (open triangles) or control mouse F(ab')\(_2\) (closed triangles) and 10 \(\mu\)g/ml LPS (open circles) and analyzed by \(\beta\)-scintillation. Data are expressed as percent increase in \(^{3}H\)-AA release compared with unstimulated samples. Data from one donor out of two tested, giving similar results, are shown. (C) Cell lysates from unstimulated, anti-CD45RA or anti-CD69 stimulated monocytes, were incubated with radiolabeled PC vesicles for 1 h at 37°C. Then phospholipids were extracted and chromatographed, and radiolabeled lyso-PC was quantitated. Results are expressed as percent increase in lyso-PC release from PC vesicles compared with unstimulated samples. Data from one donor out of two tested, giving similar results, are shown.
the wide variety of immunological, vasoactive, and metabolic effects exerted by AA metabolites on several different cell types in vivo, cross-linking of monocyte CD69 receptors in vivo is therefore expected to significantly contribute to the amplification and regulation of inflammatory processes.

**NO Production by CD69-stimulated Monocytes.** NO is a reactive nitrogen intermediate with potent and pleiotropic properties, including the capacity to directly kill parasites, tumor cells, and bacteria (22-25). NO is extremely unstable in aqueous solution, as it is rapidly oxidized to NO₂⁻ and NO₃⁻. Moreover, NO stimulates guanylate cyclase activity with consequent generation of cyclic guanylate (cGMP). NO can be indirectly measured, therefore, by quantitating NO₂⁻ accumulation and cGMP generation (26). To investigate the possibility that stimulation of CD69 in monocytes resulted in NO production, we evaluated NO₂⁻ accumulation and cGMP generation in anti-CD69-treated monocytes. Fig. 4A shows that, CD69 cross-linking with F(ab')₂ anti-CD69 plus F(ab')₂ GaM, induced increasing amounts of NO₂⁻ over time. In contrast, LPS stimulation, alone or in combination with IFN-γ (data not shown), was largely ineffective. CD69-dependent NO₂⁻ accumulation was due to NO generation by NO synthase, since NO synthase inhibitor l-NMMA completely abolished NO₂⁻ accumulation. Accordingly moreover, unlike LPS (or LPS plus IFN-γ, data not shown), CD69 stimulation caused significant cGMP generation in monocytes (Fig. 4B). In contrast to the murine system, where the induction of relevant NO production from mononuclear phagocytes is induced by two synergistic signals, including IFN-γ, IFN-α, or IFN-β plus LPS, or IFN-γ plus TNF-α or TNF-β (27, 28), human monocytes are largely unresponsive to cytokines, even in combination with LPS (29). It is interesting to note that human monocytes have been shown to produce NO only after interaction with tumor cells, but not with untransformed cells, although the receptors involved in these interactions remained obscure (29). As we observed that cross-linking of monocyte CD69 receptors induces release of high amounts of NO, this suggested that CD69 cross-linking could also trigger NO-dependent cytotoxicity programs in monocytes.

**CD69 Engagement Induces NO-dependent Cytotoxic Activity of Monocytes.** We therefore evaluated whether CD69 cross-linking was able to trigger monocyte cytotoxic activity through a NO-dependent mechanism. We found that anti-CD69 stimulation significantly increased spontaneous cytotoxicity against the L929 murine fibroblast line (Fig. 5A) and induced redirected cytotoxicity towards the P815 FcyRII⁺ murine mastocytoma cell line (Fig. 5B). It is interesting that treatment with NO synthase inhibitor l-NMMA was able to inhibit both the enhancement of spontaneous cytotoxicity and the induction of redirected cytotoxicity (Fig. 5C), indicating that NO generation is directly responsible for CD69-induced monocyte cytotoxicity. As mononuclear phagocytes play an important role in cancer-related immunological processes (30, 31), our data suggest that CD69 cross-linking would enable monocytes to exert a potent tumoricidal activity in vivo, through the production of reactive nitrogen intermediates.

**Concluding Remarks.** Primary sequence analysis and chromosomal mapping have recently allowed enlistment of CD69 in the NKC family of signal-transducing receptors (32). Members of this family, which includes the NKG2 receptors in humans, the NKR-P1 receptors in the rat, and the Ly-49...
receptors in the mouse, are expressed mainly on NK cells, share a remarkable sequence homology, similar topology, and molecular assembly. All members of the family, in fact, are disulfide-linked homodimers, formed by type II single transmembrane glycoproteins, with a C-type lectin binding domain in the extracellular portion of the molecule. Moreover, NKC family members are all able to generate an intracellular signal upon molecular cross-linking. However, a number of features clearly distinguish CD69 from other members of the family. NKG2, NKR-P1, and Ly-49 receptors, in fact, are coded for by clusters of crosshybridizing genes, some displaying allelic polymorphism and possible posttranscriptional regulation by alternative splicing, whereas CD69 is the only product of a single nonpolymorphic gene. Moreover, expression of most NKC family receptors appears restricted almost exclusively to NK cells, in a constitutive fashion. On the contrary, CD69 is expressed only upon activation of lymphocytes, including NK cells. Finally, evidence has accumulated indicating that CD69 may be expressed on other hematopoietic cell lineages (14–17).

Here we show that CD69 is constitutively expressed on human circulating monocytes and that it is likely to represent an important route to monocyte activation, as cross-linking of CD69 receptors is sufficient to induce production of key soluble mediators and to trigger NO-dependent cytotoxic activity towards transformed cell lines. Only the identification of natural CD69 ligands, however, will reveal the biological significance of monocyte CD69 expression, and the possible relevance in inflammation processes and tumor clearance in vivo.

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References

Figure 5. CD69-induced monocyte cytotoxicity. (A) Purified monocytes were incubated with 3H thymidine-labeled L929 cells at different E/T ratio for 18 h in the presence (closed triangles) or absence (open triangles) of 10 μg/ml F(ab')2 anti-CD69, or mouse F(ab')2 control mAb (closed circles), in combination with 10 μg/ml F(ab')2 GaM. 3H release in the supernatants was measured by scintillation counting. Results are expressed as mean ± SD of triplicate cultures. Data are from one representative donor out of three tested giving similar results. (B) Purified monocytes were incubated with 51Cr-labeled P815 cells at different E/T ratios for 18 h in the presence (closed triangles) or absence (open triangles) of 10 μg/ml anti-CD69, or anti-CD45RA (closed circles), 51Cr release in the supernatants was measured by gamma counting. Results are expressed as mean ± SD of triplicate cultures. Data are from one representative donor out of three tested giving similar results. (C) Monocytes were tested as above for cytolytic activity against L929 cells (E/T cell ratio, 3:1) and redirected killing versus P815 cells (E/T cell ratio, 24:1), using control mAbs as above described (black columns), anti-CD69 mAbs as above described (striped columns) or anti-CD69 mAbs together with 0.5 mM L-NMMA (shaded columns). Results are expressed as mean ± SD of triplicate cultures. Data are from one representative donor out of three tested giving similar results.


