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Syk and Lyn phosphorylation induced by FcγRI and FγRII crosslinking is determined by the differentiation state of U-937 monocytic cells

Claudia Hallal-Calleros [a], José Agramonte-Hevia [a], Claudia Garay-Canales [a], Janet M. Oliver [d], Cristhian Guerra-Araiza [b], David Heras [a], Ignacio Camacho-Arroyo [b], Isabel Soto-Cruz [c], Enrique Ortega [a,*]

[a] *Departamento de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México – UNAM, Apartado Postal 70228, Ciudad Universitaria, 04510 México, D.F., Mexico*
[b] *Facultad de Química, Universidad Nacional Autónoma de México, México, D.F., Mexico*
[c] *FES Zaragoza, Universidad Nacional Autónoma de México, México, D.F., Mexico*
[d] *Department of Pathology and Cancer Research and Treatment Center, University of New Mexico, Albuquerque, NM, USA*

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Abstract

Fcγ receptor (FcγR)-mediated phagocytosis by mononuclear phagocytes is an essential function in host defense. This process is initiated by crosslinking of membrane FcγRs, which induces phosphorylation and activation of Src and Syk tyrosine kinases. Activation of these enzymes is essential for initiating the biochemical cascade that results in the cytoskeletal and membrane changes involved in phagocytosis. Phagocytic capacity and other effector functions of mononuclear phagocytes change during differentiation/maturation of these cells. This is a complex process governed by different soluble and micro-environmental factors, giving rise to populations of cells with distinct phenotypic characteristics. Several agents, including calcitriol, have been shown to induce in vitro differentiation-related phenotypic changes in monocytic cell lines. In this paper, we characterized the changes in the initial biochemical signals associated with the increase in FcγR-mediated phagocytosis induced by calcitriol in monocytic U-937 cells. The 10-fold increase in phagocytic capacity is not accompanied by an increase in FcγR expression. However, the phosphorylation levels of Lyn and Syk after FcγRI or FcγRII crosslinking are increased after calcitriol treatment. Our results suggest that signaling induced by FcγR in mononuclear phagocytes is not only dependent on the quantity of FcγRs aggregated by a stimulus, but it is highly dependent on the cell's differentiation state.
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1. Introduction

Membrane receptors for the Fc portion of immunoglobulin G (FcγR) are a family of membrane molecules expressed on most leukocytes. Crosslinking of FcγRs by antigen–antibody complexes or opsonized particles triggers various responses such as phagocytosis, release of pro-inflammatory molecules, cytokine production and antibody-mediated cell cytotoxicity.

Three distinct classes of FcγRs (FcγRI, FcγRII and FcγRIII) are recognized, based on their affinity for IgG, protein and gene structure, and cell distribution. Each class of FcγRs consists of several individual receptor isoforms (reviewed in [1]). Human FcγRI (CD64) is expressed on monocytes and macrophages as a heterotrimer comprising an α subunit non-covalently associated to a pair of disulfide-linked FcRγ chains. The α subunit contains the IgG binding site, while each γ chain contains a consensus Immunoreceptor Tyrosine-based Activation Motif (ITAM) essential for cellular activation [1,2]. Three human FcγRII genes (A, B and C) encode monomers (CD32) with almost identical extracellular

* Corresponding author. Tel.: +52 5622 3834; fax: +52 5622 3369.
E-mail address: ortsoto@servidor.unam.mx (E. Ortega).

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IgG binding domains, a single transmembrane domain and distinct cytoplasmic tails. FcγRII isoforms A and C contain in their cytoplasmic domain activating ITAM motifs, while FcγRII B contain inhibitory ITIM motifs. FcγRIIa and FcγRIIc are expressed on monocytes, macrophages, granulocytes, platelets and NK cells, while FcγRIIb is expressed on monocytes, T cells and B cells [3,4]. FcγRIII (CD16) has two isoforms: the A isoform is a transmembrane protein expressed in macrophages associated to a pair of ITAM-containing FcRγ chains. The FcγRIIIB isoform, expressed exclusively on neutrophils, is anchored to the cell membrane by a GPI linkage, and lacks transmembrane and cytoplasmic domains.

The activatory FcγRs (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIA) share a similar mechanism of signal transduction on monocytic cells: clustering of activatory FcγRs induces the immediate phosphorylation of ITAM tyrosines, usually catalyzed by the Src family tyrosine kinase Lyn [3,5,6]. The tyrosine-phosphorylated ITAMs serve as binding sites for the SH2 domains of the tyrosine kinase Syk. Binding to phosphorylated ITAM results in activation of Syk catalytic activity, enabling it to phosphorylate downstream signaling proteins [6–10]. The essential role of Syk in connecting early FcγR signaling to effector functions has been demonstrated by several approaches, which have unequivocally shown that Syk kinase activation is necessary for all the studied responses mediated through FcγRs [9]. Thus, FcγR mediated phagocytosis is inhibited by the Syk specific inhibitor piceatannol [11,12], and by inhibition of Syk expression by antisense oligonucleotides [13]. It has also been shown that macrophages from $Syk^{-/-}$ mice are unable to internalize IgG-opsonized particles [14].

Monocytes leave the bloodstream and enter tissues where they differentiate into macrophages. During this process, monocytic cells undergo several phenotypic changes that increase some capabilities while down-regulate others [15]. Monocytic cell lines can be induced to differentiate in vitro by several agents, including the active metabolite of Vitamin D3 (1α,25-di-hydroxy-Vitamin D3 or calcitriol). Differentiation of U-937 cells with calcitriol has been reported to modify the expression of complement receptor type 3 (CR3) and to mediate an increase in the number of the cell's IgG-binding sites and an increase in the phagocytosis of IgG-opsonized particles [16]. In order to explore the biochemical basis of the differentiation-related increase in FcγR-mediated phagocytosis, we have comparatively examined the expression level of FcγRs isoforms, as well as the activation of Lyn and Syk kinases in response to FcγR crosslinking in U-937 monocytic cells before and after calcitriol-induced differentiation. We found that in U-937 cells calcitriol induced a 10-fold increase in FcγR-mediated phagocytosis that is not accompanied by a similar increase in FcγR expression. The levels of Lyn and Syk phosphorylation induced by FcγR crosslinking are increased, and while Syk expression is not modified, Lyn expression is higher after differentiation. These results show that Lyn and Syk are differently regulated in monocytic cells in distinct states of maturation. Our data demonstrate that signaling through a single class of FcγR can vary depending not only on the magnitude of the stimulus but also on the differentiation state of the cell.

2. Materials and methods

2.1. Chemicals

1α,25-Di-hydroxy-Vitamin D3 (VD3) was from Calbiochem (La Jolla, CA). Protein A agarose was from GIBCO Laboratories (Grand Island, NY). Anti-phosphotyrosine (PY20-HRP), anti-Syk (LR) and anti-Lyn (Lyn 44) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Lyn monoclonal antibody (mAb) was from Transduction Laboratories. Fab fragments of the 32.2 (anti-FcγRI) and IV.3 (anti-FcγRII) mAbs were obtained in our laboratory from the purified antibodies using immobilized ficin for 32.2 or immobilized pepsin (Pierce, Rockford, IL) for IV.3, following the manufacturer's instructions. Secondary anti-rabbit IgG-HRP and anti-mouse IgG $(Fab')_2$ fragments were purchased from Jackson Immunoresearch (Amish, PA). Reagents for RNA isolation and RT-PCR were from GIBCO-BRL, Inc. (Gaithersburg, MD) and Sigma Chemical Corp. (St. Louis, MO). Taq DNA polymerase was purchased from Perkin-Elmer (Branchburg, NJ).

2.2. Cell culture and in vitro differentiation

U-937 cells (obtained from ATCC) were grown in RPMI 1640 medium with 10% heat inactivated fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 0.1 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Differentiation was induced by culturing $(0.5–1) \times 10^6$ cells/ml with 100 nM calcitriol for 72 h. After treatment, adherent cells were detached by gently pippeting and both adherent and non-adherent cells were used for experiments.

Hybridomas producing the 32.2 and IV.3 mAbs were obtained from ATCC. Antibody was purified from culture supernatants by chromatography in protein A agarose (IV.3) or protein G agarose (32.2) columns.

2.3. Flow cytometry

For flow cytometry, 1×10^6 U-937 cells/ml were incubated in PBS-5% FBS with 10 μg/ml of specific antibodies: IV.3 (anti-FcγRII), 32.2 (anti-FcγRI), 3G8 (anti-FcγRIII), UCHM-1 (anti-CD14), 2LMP19c (anti-CD11b), or an isotype-matched control antibody, for 60 min at 4 °C. Cells were washed three times with the same buffer and stained with 200 μl of a 1:200 dilution of a rabbit anti-mouse IgG coupled to FITC (Zymed, San Francisco, CA). After washing, cells were fixed with 1% paraformaldehyde (PFA) for

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