Signaling mechanism of integrins, Fc receptors and G-protein coupled receptors in neutrophils

Ph.D. thesis

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1. Introduction

Neutrophils play an essential role in the destruction of invading microorganisms and in the pathogenesis of several autoimmune diseases. In the course of neutrophil antibacterial functions superoxide production and degranulation result in killing of invading microorganisms. The activation of the cells is mediated by several different receptors. In my Ph.D. work, the signaling mechanism of integrins, Fc receptors and G protein coupled receptors were investigated in neutrophils.

Both integrin and nonintegrin receptors are required for neutrophil activation in the inflamed environment. Prior findings suggested that the nonintegrin signals are only required to increase the binding capacity of integrins, while the eventual cellular responses are mediated by integrin crosslinking upon their ligand binding (i. e., suggesting a serial hierarchical relationship between the two signals). This hypothesis was based on the fact that neutrophil activation by anti-integrin antibodies results in full activation of the cells without any additional stimulus, indicating that integrin crosslinking by itself is sufficient for full activation of neutrophils. In the first part of my Ph.D. work we attempted to

determine how is adhesion dependent activation of neutrophils is mediated, what is the relative contribution of integrin and non-integrin signals to cell responses, and what is the relationship between these two signals.

Activation of neutrophils through Fc receptors have crucial role in the destruction of invading microorganisms and in the pathogenesis of several autoimmune diseases. Neutrophils are responsible for significant fraction of tissue damage in autoimmune diseases. However, the signaling mechanism of Fc receptors was incompletely understood. In the second part of my experiments, the mechanism of Fc receptor signaling was investigated in neutrophils.

My supervisors and their coworkers proved by using wide-specificily inhibitors that tyrosine kinases play critical role in effector responses induced through G-protein-coupled fMLP receptor. It was, however, not known which kinase pathways play crucial role in cell responses induced by fMLP. In the third part of my Ph.D. work, the contribution of tyrosine kinase signaling pathways to G protein coupled receptor signaling was investigated.

2. Objectives

In our experiments we investigated in neutrophils:

1., what is the relationship between integrin and non-integrin signals in the course of adhesion dependent activation: is crosslinking of integrins sufficient to induce full activation of neutrophils?

2., which signaling steps are critical for the Fc receptor function?

3., what are the role of Src-family tyrosine kinases, MAP-kinases and Syk in G-protein-coupled receptor signaling?

3. Methods

Animals, bone marrow chimeras: In our experiments Fc receptor γ-chain; Fcγ-receptor III; Hck/Fgr/Lyn and Syk deficient mice were used. Investigation the role of Syk in signaling required a special approach, because $Syk^{-/-}$ mice die perinatally. To overcome this problem, we generated bone marrow chimeras by using the fetal liver of the $Syk^{-/-}$ embryos and investigated $Syk^{-/-}$ neutrophils from these chimeras.

Neutrophil preparation: Mouse neutrophils from bone marrow, human neutrophils from volunteers venous blood were isolated by gradient centrifugation.

Neutrophil stimulation: Adherent neutrophils were stimulated by immobilized anti-integrin antibodies, by TNF on fibrinogen surface or by plate bound immune complexes. Cells in suspension were activated by G-protein-coupled receptor agonists (fMLP, MIP-2).

Superoxide release assay: Superoxide production of neutrophils was determined by cytochrome c reduction test spectrophotometrically.

Cell adhesion assay: Cells were stimulated on an adhesion surface. Following removing suspended neutrophils the number of cells that remained adherent was quantitated by an acid phosphatase assay spectrophotometrically.

Degranulation assay: Granule release markers were measured from the supernatant of activated neutrophils. Exocytosis of primary granule marker β-glucuronidase was determined by a fluorometric assay, release of secondary granule marker lactoferrin and secretory vesicles human serum albumin marker were measured by ELISA.

Flow cytometry: Cell surface expression of proteins was detected by flow cytometry.

Measurement of calcium signals: Calcium signal was detected by FURA2 dye using a fluorimeter.

Intracellular signaling: After stimulation lysates were prepared from neutrophils by using a Triton-based lysis buffer. Phosphorylation of Syk kinase was determined by immunoprecipitation and immunoblotting. Phosphorylation of Fc receptor γ-chain was detected by GST-Syk$(SH2)_2$ precipitation. Additionally we used immunblot technique to show the activation of p38 and ERK MAP kinase and the detection of tyrosine phosphorylation.

4. Results and conclusions

My Ph.D. thesis focuses on signal transduction in neutrophils. I characterized the responses induced by anti-integrin antibodies, the mechanism of adhesion dependent stimulation, the signaling pathways of Fc receptors and the role of kinases in G-protein-coupled receptor signaling.

In the first part of my Ph.D. work, using gene deficient (knockout) mice, blocking antibodies and antibody Fab fragments we showed that low affinity Fcγ receptors are indispensable for neutrophil responses induced by anti-integrin antibodies. These findings argue against the earlier hypothesis that crosslinking of integrins is sufficient for full activation of neutrophils, and suggest that integrin and nonintegrin signals function in a parallel fashion. Furthermore, we showed that the different nonintegrin signals (TNF, Fc receptor) are interchangeable and likely converge on the p38 MAP kinase.

In the second part of my experiments, the mechanism of Fc receptor signaling was investigated in neutrophils. Using gene deficient (knockout) mice and a newly set up activation system, we found that Src family tyrosine kinases, the Fc receptor γ chain, Syk and the p38 MAP kinase are indispensable for neutrophil responses induced by immune complexes that act through Fc-receptors. These proteins could be placed into a signaling pathway where Src family tyrosine kinases phosphorylate the Fc receptor γ chain, which then recruits Syk, eventually leading to activation of the MAP kinases.

In the third part of my Ph.D. work, the contribution of tyrosine kinase signaling pathways to G protein coupled receptor signaling was investigated. Using pharmacological inhibitors and gene deficient (knockout) mice, we showed that the p38 MAP kinase activated via Src-family kinases is indispensable for the degranulation induced by the bacterial tripeptide fMLP. Using Syk deficient neutrophils, we also disproved prior assumptions that Syk is crucial for G protein coupled receptor (e. g. fMLP, chemokine) signaling.

These results help us to understand the working paradigms of the immune system, and they may point to possible novel targets for pharmacological control of autoimmune diseases.

5. List of publications

I. ***Jakus Z****, Berton G, Ligeti E, Lowell CA and Mócsai A.*
Responses of neutrophils to anti-integrin antibodies depends on costimulation through low affinity FcγRs: full activation requires both integrin and nonintegrin signals.
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II. *Mócsai A,* ***Jakus Z****, Vantus T, Berton G, Lowell CA, Ligeti E.*
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J Immunol. 2000 Apr 15;164(8):4321-31.
IF: 6,834

III. *Mócsai A, Zhang H,* ***Jakus Z****, Kitaura J, Kawakami T and Lowell CA.*
G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells.
Blood. 2003 May 15;101(10):4155-63.
IF: 10,12

IV. *Fodor Sz,* ***Jakus Z*** *and Mócsai A.*
ITAM-based Signaling beyond the Adaptive Immune Response
Immunol Lett. 2006 Apr 15;104(1-2):29-37.
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