

## Interleukin 8 and Cell Migration to Inflammatory Sites: the Regulation of Focal Adhesion Kinase under Conditions of Migratory Desensitization

Efrat Cohen-Hillel PhD, Ilana Yron PhD, Tsipi Meshel MSc and Adit Ben-Baruch PhD

Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

**Key words:** chemokines, interleukin 8, focal adhesion kinase, actin filaments, integrins

### Abstract

**Background:** Interleukin 8 is a prototypical inflammatory chemokine that induces leukocyte migration to inflammatory sites. Leukocyte recruitment in response to gradients of this chemokine is attenuated at advanced stages of inflammation to prevent damage to surrounding healthy tissues. Our published studies suggest that over-phosphorylation of focal adhesion kinase in migration-desensitizing conditions is involved in cessation of cell motility. This over-phosphorylation of FAK was induced by IL-8 only when the receptor transmitting the chemokine signals was CXCR2 and not CXCR1, indicating that the two IL-8 receptors diverge in their signaling properties.

**Objectives:** To analyze the regulation of FAK in CXCR2-expressing hematopoietic cells under conditions of migratory desensitization, focusing on the roles played by adhesion-related components in this process.

**Methods:** Under conditions of migratory desensitization, we determined IL-8-induced cell spreading and FAK localization following disruption of actin filaments, and evaluated the role of integrins in FAK phosphorylation.

**Results:** The disturbance of intact activity of actin filaments resulted in inhibition of cell spreading and modification of FAK intracellular localization upon IL-8 stimulation. Also, adhesion-dependent pre-stimulation of integrins was required for IL-8-induced FAK phosphorylation.

**Conclusions:** Intact actin filaments and integrins are required for optimal IL-8-induced FAK phosphorylation in conditions of migratory desensitization. These observations suggest that lack of adequate activity/regulation of adhesion-related components may give rise to FAK activities that are not appropriately controlled, possibly leading to pathological conditions that are associated with perturbed leukocyte migration phenotypes.

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Interleukin 8 (CXCL8) is a highly potent inflammatory chemokine that induces the recruitment of leukocytes, primarily neutrophils, to sites of inflammation. Upon tissue damage or infectious insult, the chemokine is released by a variety of cell types. In response to chemokine gradients, leukocytes that express the corresponding IL-8 receptors CXCR1 and CXCR2 migrate to the

damaged/infected tissue and remove the invading pathogen. Thus, IL-8 activities are required for keeping immune integrity and for protection against pathogenic insults [1,2].

The motility of leukocytes in response to IL-8 is a tightly regulated process. Under physiological conditions, when the inflammatory process is active for some time and the chemokine concentrations are elevated as a result, the inflammatory process is stopped in order to prevent damage to neighboring healthy cells and to allow tissue recovery. Restraining this inflammation is mediated by different mechanisms that desensitize cellular responses to the chemokine, resulting in leukocyte migration arrest [3-6]. When inappropriately controlled, cell migration persists, giving rise to a continuous flux of leukocytes and to inflammatory diseases, such as chronic obstructive pulmonary disease and acute respiratory distress syndrome [7,8].

The involvement of IL-8 in the pathogenesis of inflammatory diseases emphasizes the need for better understanding of the mechanisms regulating cell motility in response to the chemokine. A key regulator of migration is the protein tyrosine kinase focal adhesion kinase, which is activated by its phosphorylation on six potential tyrosine phosphorylation sites. Optimal activation of FAK is required for migratory processes to take place. Moreover, FAK serves as an on/off regulator of migration, and thus its over-activation and alternatively its reduced stimulation may give rise to perturbation of cell migration [9-12].

Our overall aim was to provide insight into the regulation of FAK activation upon IL-8 stimulation. Since the IL-8 receptors CXCR1 and CXCR2 were shown to be differently regulated at many levels [13-15], we used cells from the hematopoietic lineage that express either CXCR1 or CXCR2 separately from each other. Our studies indicated that FAK is required for IL-8-induced migration; moreover, stimulation by IL-8 in conditions that activate migration (namely, exposure to relatively low IL-8 concentrations of 50 ng/ml) gave rise to significant FAK phosphorylation [16]. Our studies characterized key regulatory pathways in the control of FAK activation, showing that the phosphorylation of FAK requires intact activity of actin filaments and microtubules [17]. In addition, our results identified signaling differences between the two IL-8 receptors, CXCR1 and CXCR2, as indicated by the

FAK = focal adhesion kinase  
IL-8 = interleukin 8

finding that adhesion-dependent pre-activation of integrins is required for IL-8-induced FAK activation only in CXCR2-expressing cells, but not in CXCR1-expressing cells.

Further analyses were performed to study the regulation of FAK under conditions of migratory desensitization induced by cell exposure to relatively high IL-8 concentrations (1000 ng/ml) [3-5,18]. Our published findings indicate that under these conditions, FAK is over-phosphorylated upon IL-8 stimulation; however, this is correct only when the receptor that transmits the IL-8 signals is CXCR2 [16]. These results have two important indications: Firstly, they provide additional evidence of the functional difference between CXCR1 and CXCR2, suggesting that in cells expressing both receptors it is the equilibrium between them that eventually determines the actual activity of the cells. Secondly, over-activation of FAK may be of major importance for cessation of migration: it may result in increased adhesion of the cells, perturbing cycles of attachment and detachment that are required for optimal cell migration in response to the chemokine.

In the present study we wished to gain better understanding of the regulation of FAK in desensitizing conditions by focusing on cells expressing CXCR2, as these cells show a unique regulation of FAK under migration-attenuating conditions. Since cytoskeletal elements and integrins were shown to be involved in IL-8-induced FAK phosphorylation under conditions of migratory activation [17], we questioned whether they are associated with and participate in the regulation of FAK activation under conditions of migratory desensitization.

Our findings indicate that when desensitizing signals of IL-8 are transmitted via CXCR2, actin filaments are required for optimal cell spreading and for appropriate intracellular localization of FAK. Moreover, adhesion-dependent pre-stimulation of integrins is a prerequisite for IL-8-induced FAK phosphorylation under these conditions. Together, the findings of the present study shed light on the regulation of FAK in migration-desensitizing conditions in which FAK over-phosphorylation may participate in cessation of leukocyte migration to inflammatory sites.

## Materials and Methods

### Cells

Rat basophilic leukemia 2H3 cells, stably expressing CXCR2 (namely CXCR2-RBL cells), were produced as previously described [17]. Over 85% of the cells expressed CXCR2, while untransfected cells did not express IL-8 receptors or bind IL-8 (our unpublished results and [19]).

### Confocal analyses

CXCR2-RBL cells were grown overnight in starvation medium [17]. Prior to the assay, the cells were pre-exposed to 1  $\mu$ M cytochalasin D (Sigma, St. Louis, MO, USA), diluted in dimethyl sulfoxide, for 1 hour at 37°C. The cells were then exposed to IL-8 (PeproTech, Rocky Hill, NJ) for 5 min at 37°C, followed by

fixation, permeabilization, blocking and staining of FAK and actin, as previously described [16,17]. The localization of FAK was determined by rabbit polyclonal antibodies against FAK (sc-558; Santa Cruz Biotechnology, Santa Cruz, CA), followed by staining with rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Actin arrangement was determined by staining the cells with phalloidin-FITC (Alexa 488, Molecular Probes, PoortGebouw, The Netherlands).

Cells were analyzed using a confocal microscope (Zeiss, Oberkochen, Germany), equipped with 25 mW Ar 488 nm laser and 1 mW HeNe laser 543 nm. 63XNA1.4 plan-apochromat oil-immersion lens, red fluorescence long-pass filter 560 nm, and green fluorescence band-pass filter 505-550 nm were used for all imaging.

### Determination of FAK phosphorylation

**FAK phosphorylation in adherent RBL cells:** The cells were grown as previously described [16], then stimulated by IL-8 (PeproTech). In specific experiments the cells were pre-exposed to wortmannin (1000 nM, Sigma) for 1 hour at 37°C. The cells were scraped and lysed in RIPA lysis buffer, followed by immunoprecipitation and Western blotting as previously described [16]. Briefly, the immunoprecipitation was performed with rabbit anti-FAK antibodies (Santa Cruz Biotechnology), protein expression was determined by antibodies against FAK (same as above), and phosphorylation by monoclonal murine antibodies against phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY). Bands on immunoblots were quantitated by densitometry.

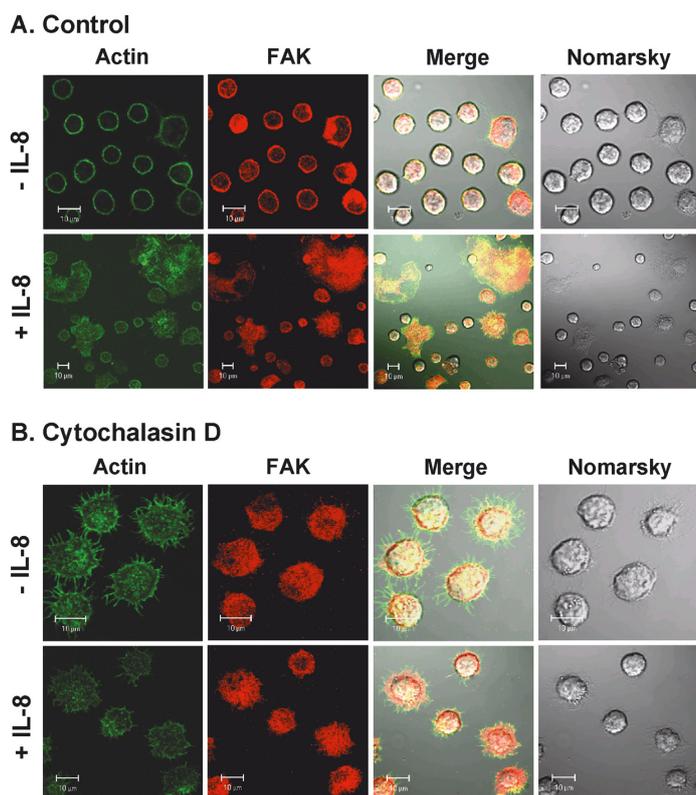
**FAK phosphorylation in suspended RBL cells:** Cells were kept in suspension overnight, and were viable and functional, as previously described [17]. The cells were pelleted and resuspended in medium containing IL-8 or in medium alone. FAK phosphorylation was determined as described above.

## Results

Adhesion is a prerequisite for intact cellular migration, requiring participation of cytoskeletal elements, including actin filaments [20-22]. We previously demonstrated that actin filaments are required for optimal phosphorylation of FAK, not only in conditions of migratory activation but also when the cells are exposed to IL-8 in desensitizing settings [17]. In order to provide further insight into the way that FAK is regulated in the context of CXCR2 in desensitizing conditions, we determined the involvement of actin filaments in IL-8-induced cell spreading and in FAK cellular localization under migration-desensitizing settings. To this end, CXCR2-RBL cells were exposed to 1000 ng/ml IL-8, with or without pre-treatment with cytochalasin D, a specific inhibitor of actin polymerization. The ability of the cells to spread in response to chemokine stimulation and the localization of FAK and actin filaments were compared to control cells (treated by DMSO, the solubilizer of CD).

RBL = rat basophilic leukemia  
IgG = immunoglobulin G

DMSO = dimethyl sulfoxide  
CD = cytochalasin D

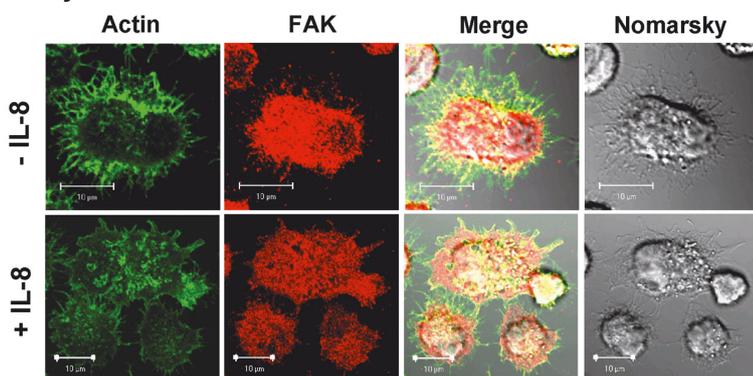


**Figure 1.** Disruption of actin integrity results in perturbation of cell spreading in response to IL-8 and in modified localization of FAK. The localization of FAK and actin filaments in adherent CXCR2-RBL cells was determined prior to and following stimulation by 1000 ng/ml IL-8 for 5 min at 37°C. **[A]** Control cells treated by DMSO, the solubilizer of cytochalasin D. **[B]** Cells pre-treated by cytochalasin D for 1 hour, at 37°C. The drug did not affect the expression levels of CXCR2 by the cells, nor did it affect cell viability. Localization of FAK was determined by rabbit polyclonal antibodies against FAK, followed by staining with rhodamine-conjugated goat anti-rabbit immunoglobulin G. Actin arrangement was determined by staining the cells with phalloidin-FITC. -IL-8 = cells that were not stimulated by IL-8. +IL-8 = cells stimulated with IL-8. A representative experiment of n=3-4 is presented.

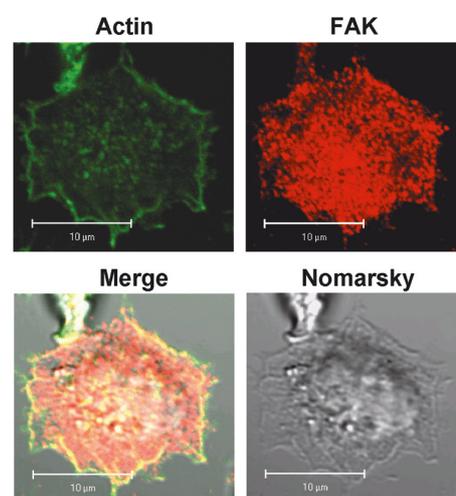
Figure 1 shows that in control cells, prior to IL-8 stimulation, the actin cortex was localized at the cell periphery. In this region, FAK showed a thin line of overlap with the actin filaments [Figure 1A]. When the control cells were stimulated by the chemokine, a highly prominent cell spreading was induced, accompanied by enriched actin network and definite FAK co-localization with the actin filaments, including cell regions that form contact with the substratum below [Figure 1A].

In contrast, a totally different phenotype was observed in cells treated by CD [Figure 1B]. Firstly, upon IL-8 stimulation, the spreading of the CD-treated cells was perturbed, when compared to cells exposed to IL-8 alone (namely, without disruption of actin organization by CD) [Figure 1B, 2A and 2B compared to Figure 1A]. The CD + IL-8-treated cells had a condensed nucleus, the actin cortex was broken into non-organized protrusions, and FAK was localized in loosely spread areas that contoured the nucleus [Figure 1B, 2A and 2B]. Secondly, in cells that were treated with CD alone, without IL-8, prominent perturbations of the actin cortex were observed [Figure 1B and 2A]. In these cells there was no localization of FAK in the spiky protrusions that included actin filaments. This phenotype of the cells was somewhat relieved by the chemokine, showing a certain degree of spreading [Figure 1B, 2A and 2B]. However, as noted in the first premise above, this spreading was far from being normal, deviating immensely from the extensive spreading noticed in cells that were not treated by CD but were stimulated by IL-8 [Figure 1B, 2A compared to Figure 1A].

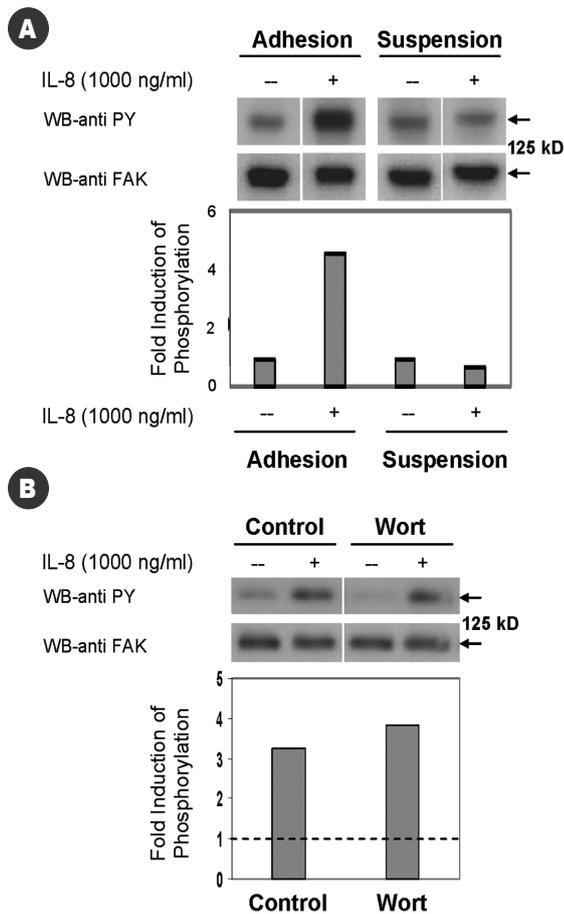
### A. Cytochalasin D



### B. Cytochalasin D



**Figure 2.** Actin and FAK localization in cells stimulated by IL-8 after disruption of actin filaments. The localization of FAK and actin filaments in adherent CXCR2-RBL cells treated by cytochalasin D for 1 hour at 37°C is shown in higher magnification than in Figure 1. The cells are shown before **[A]** and after **[A, B]** stimulation by 1000 ng/ml IL-8 for 5 min at 37°C. The drug did not affect the expression levels of CXCR2 by the cells, nor did it affect cell viability. The localization of actin filaments and FAK was determined as described in Figure 1. -IL-8 = cells that were not stimulated by IL-8. +IL-8 = cells stimulated with IL-8. A representative experiment of n=3 is presented.



**Figure 3.** IL-8-induced FAK phosphorylation in conditions of migratory desensitization requires adhesion-dependent pre-stimulation of integrins. **[A]** IL-8-induced FAK phosphorylation in CXCR2-RBL adherent cells compared to cells grown in suspension. The cells were stimulated by 1000 ng/ml IL-8 for 5 min at 37°C. The basal level of phosphorylation without IL-8 stimulation was given the value of 1. A representative experiment of n=5 is presented. **[B]** FAK phosphorylation in response to 1000 ng/ml IL-8 was determined in adherent CXCR2-RBL cells treated by DMSO (the solubilizer of wortmannin), or 1000 nM wortmannin (Wort) for 1 hour at 37°C. The basal level of phosphorylation without stimulation was given the value of 1. The drug did not affect the expression levels of CXCR2 by the cells, nor did it affect cell viability. A representative experiment of n=5 is presented.

Together, these results indicate that actin filaments play a key role in IL-8-induced adhesion, and that in their absence the IL-8-induced spreading characteristics of the cells are perturbed. However, stimulation by the chemokine could partly relieve the constraints that were induced by disrupting actin integrity (by the CD treatment). This is evidenced by the ability of the chemokine to cause partial spreading, which is accompanied by reorganization – albeit not normal – of FAK. In addition, the findings of this analysis indicate that associations exist between FAK and actin filaments, and that the interactions between these two components are modified upon IL-8 stimulation.

Evidently, the above results demonstrate the ability of IL-8, as a prototypical chemokine, to act as a key regulator of cell

adhesion under migration-desensitizing conditions. Adhesion is a highly coordinated process that is mediated by the activation of integrins [22,23]. Our published results [17] indicate that integrins are prerequisites for IL-8-induced FAK activation under conditions that induce cell motility, thus raising the question whether integrins are required also for FAK activation when stimulation by the chemokine leads to desensitization of migration.

To explore this issue, we neutralized adhesion-dependent integrin-stimulating signals by growing the cells in suspension, and compared the responses of such cells to cells grown in adhesion, where integrins are stimulated by contact with extracellular matrix proteins. Under suspension conditions, which abolish all adhesion-dependent signals, the cells were stimulated by 1000 ng/ml IL-8 and FAK phosphorylation was determined. As shown in Figure 3A, no FAK phosphorylation was induced by IL-8 stimulation in suspended cells. These results are in marked contrast to the high levels of FAK phosphorylation that were detected in IL-8-stimulated control cells that were adherent.

The above findings indicate that adhesion-dependent pre-stimulation of integrins is essential for IL-8-induced FAK activation in conditions that desensitize cell migration. Major connecting factors between the different factors that were shown here to regulate IL-8-induced FAK phosphorylation, namely the actin cytoskeleton and integrin stimulation, are phosphatidylinositol kinases [24]. These enzymes are known to be activated by IL-8 [25], and therefore we investigated whether they are required for IL-8-induced FAK phosphorylation. The results shown in Figure 3B, in which phosphatidylinositol kinases were inhibited by wortmannin, suggest that this family of kinases is not involved in the signaling pathways leading from IL-8 stimulation to FAK phosphorylation.

## Discussion

The findings of the present study supplement those of our previous investigations on the mechanisms regulating IL-8-induced migration. Our published results indicate that under conditions of migratory desensitization, signals that are transmitted by the chemokine via the CXCR2 receptor lead to elevated levels of FAK phosphorylation [16]. This over-activation of FAK may give rise to increased adhesion, which interferes with the ability of the cells to undergo cycles of attachment and detachment that are required for cell motility. This potential mechanism of desensitization may come into effect together with other attenuating mechanisms, e.g., phosphorylation of serine and threonine residues on the carboxyl terminus of the receptor, a process known to result in uncoupling of heterotrimeric G proteins from the receptor and, consequently, in functional desensitization [5,6].

Our published research indicates that under migration-desensitizing conditions, actin filaments as well as microtubules are essential for FAK activation [17]. The present study shows that in the same conditions, perturbation of actin polymerization results in inappropriate spreading and localization of FAK upon stimulation by the chemokine. In addition to cytoskeletal elements, adhesion-stimulated integrins were found to be essential for IL-8-induced FAK phosphorylation in CXCR2-expressing cells in conditions of migratory desensitization. Of note, integrins were

found to be prerequisites for FAK phosphorylation also when the cells were exposed to IL-8 in migration-activating conditions [17]. Therefore, it is possible that *in situ*, leukocytes that approach the inflammatory site are regulated by extracellular matrix proteins that are present at the site; therefore, signals that are transmitted by integrins are required for adequate FAK regulation under both conditions – activation and desensitization of migration.

It should be emphasized that the need for adhesion-dependent integrin pre-stimulation for IL-8-induced FAK phosphorylation was evident only when the signals of the chemokine were transmitted via CXCR2, and not via its other receptor, CXCR1 [17]. Moreover, elevated FAK phosphorylation in conditions of desensitization was noted only when the receptor transmitting the IL-8 signals was CXCR2 and not CXCR1. However, many of the target cells that respond to IL-8 express both receptors together. In the inflammatory context, neutrophils constitute the major cell population that responds to IL-8, via both CXCR1 and CXCR2 [1,2,13]. Therefore, the functional response of neutrophils to IL-8, including migration to and at the inflammatory site, actually reflects the equilibrium that exists between signals that are transmitted by both receptors together. Eventually, the differences in the signaling pathways that are induced by the two IL-8 receptors, CXCR1 and CXCR2, result in tight regulation of the responses of the target cells to the chemokine. Such mechanisms may be crucial for regulating cell migration *in vivo*, as their appropriate levels of activity enable not only potent migration of leukocytes at the initial stages of inflammation, but also cessation of migration when the inflammatory process should be shut-off.

Overall, when inappropriately regulated, the mechanisms of FAK activation may give rise to perturbed phenotypes of leukocyte migration – at the levels of activation and/or desensitization. Skewing from the normal phenotype of leukocyte motility may be responsible, at least partly, for exacerbated or deficient recruitment of these cells to sites of inflammation, as evidenced by a variety of pathological conditions. For example, it is possible that such impaired regulation of FAK activation following IL-8 stimulation gives rise to inflammatory diseases in which leukocyte migration to damaged/infected sites is not halted despite the relatively high concentration of the chemokine that is present at these sites.

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**Correspondence:** Dr. A. Ben-Baruch, Dept. of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv 69978, Israel.  
Phone: (972-3) 640-7933; Fax: (972-3) 642-2046  
email: aditbb@tauex.tau.ac.il