

Tec kinase stimulates cell survival in transfected Hek293T cells and is regulated by the anti-apoptotic growth factor IGF-I in human neutrophils

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ABSTRACT

Objective: Previously, we showed that the phosphatidylinositol-3 kinase (PI₃K) pathway mediates the anti-apoptotic effects of IGF-I in human neutrophils independently of its down-stream target Akt. In this study, we investigated whether IGF-I regulates Tec kinase, an alternative down-stream target of PI₃K, in neutrophils and whether this molecule is able to affect apoptosis.

Design: We investigated the translocation of Tec kinases in neutrophils after stimulation with IGF-I. Furthermore, we transiently and stably transfected Hek293T cells with constructs expressing different forms of Tec kinase and measured the level of cell survival and apoptosis/necrosis through trypan blue exclusion test and Annexin-V/propidium iodide labelling, respectively.

Results: We show that IGF-I stimulates the translocation of Tec kinase to the membrane in neutrophils in a PI₃K dependent manner. Overexpression of Tec kinase augments cell survival by inhibition of necrosis. The pro-survival effect is attenuated by the deletion of the kinase domain but not by inactivation of this domain by a single amino acid substitution.

Conclusion: Tec kinase can act as a prosurvival factor and is regulated by IGF-I in human neutrophils through PI₃K activation.

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1. Introduction

Previously, we showed [1] through inhibitor studies that the phosphatidylinositol-3 kinase (PI₃K) pathway, but not the MEK-ERK pathway mediates the anti-apoptotic effects of IGF-I in human neutrophils, and that the signalling mechanism of IGF-I is different from the one used by GM-CSF, another PI₃K dependent anti-apoptotic agent. IGF-I did not operate through Akt in contrast to GM-CSF which stimulated this process in a PI₃K dependent-manner [2]. PI₃K has several downstream targets other than Akt which may be involved in inhibition by IGF-I of neutrophil apoptosis, including Tec family kinases.

In 1993, the importance of Tec family kinases came into light when several research groups discovered that a point mutation in the gene encoding one Tec family member, Btk (Bruton's tyrosine kinase), leads to immunodeficiency diseases in humans (X-linked agammaglobulinemia (XLA)) and mice (X-linked immunodeficiency (Xid)) [3]. Tec family kinases are not only expressed in hematopoietic cells

but also outside of the hematopoietic lineage. For instance, Tec kinase was initially discovered in hepatocellular carcinoma, but was subsequently found to be expressed in all hematopoietic cells and in normal liver and kidney cells [4].

Tec kinases belong to a non-receptor tyrosine kinase family consisting of at least 5 members, and three of them are expressed by neutrophils (Tec, Btk and Bmx) [5]. Tec family kinases are abundantly expressed in the cytoplasm and predominantly unphosphorylated (inactivated) in unstimulated cells (resting state). Before acting as a tyrosine kinase protein, Tec kinase has to be translocated to the membrane, which involves the phosphorylation of PIP₂ by PI₃K and subsequent recruitment of Tec kinase to PIP₃ through its pleckstrin homology (PH) domain [6]. Subsequently, Tec kinase can be activated by phosphorylation of tyrosine residues by members of the Src-kinase family, including Lyn and Fyn kinase resulting in an active kinase.

In neutrophils, translocation of Tec kinase is induced by the chemoattractant fMLP, by the cell wall component of gram⁻ bacteria lipopolysaccharide (LPS) and by cross linking of the Fc receptor CD16b via activation of PI₃K [5,7,8]. Furthermore, Tec kinase can be activated in other cell types in response to ligation of antigens to the BCR (B-cell receptor) [9] and TCR (T-cell receptor) [10] and a wide range of other stimuli including growth factors (hepatocyt growth factor (HGF) [11], macrophage colony stimulation factor (M-CSF) [12], granulocyte colony stimulation factor (G-CSF) [13], granulocyte-macrophage colony stimulation factor (GM-CSF) [14], epidermal growth factor (EGF) [15], stem

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cell factor (SCF) [16]) and cytokines ((interleukin (IL) 3 [17], IL-6 [18], and IL-8 [5])).

Aside from functional roles of Tec kinase in regulation of cell proliferation, expression of cytokines, migration, phagocytosis and differentiation, Tec kinase and especially the other members of the Tec kinase family are involved in the regulation of cell survival. For instance, Melcher et al. [12] found that there was an impaired survival rate in Tec^{-/-}Btk^{-/-} macrophages compared to the wild-type macrophages. Although the Tec^{-/-} and BTK^{-/-} macrophages showed a small reduction in number of cells, the combined knockout showed a 75% reduction in cell numbers. There is a further lack of direct evidence for the involvement of Tec kinase in the regulation of cell survival, but studies of other Tec kinase family members strongly suggest that they can regulate apoptosis. Mutations in the Btk gene are believed to lead to inappropriate apoptotic cell death of pre-B-cells leading to XLA [19,20]. Accordingly, in the murine variant of the disease (Xid), in contrast to wild-type cells, Xid B-cells underwent apoptotic cell death after stimulation with anti-IgM [21]. More recently, Honda et al. [22] showed that neutrophils from patients with XLA showed a significantly higher percentage of apoptotic cells after stimulation with TNF α and fMLP compared to control neutrophils. Furthermore, Petro et al. [23] provided evidence that in mice splenocytes, Btk targets NF- κ B to activate the Bcl-x promoter through PLC γ 2 and Vassilev et al. [24] described that in chicken B-lymphoma cells (DT40) Btk binds to Fas receptor where it prevents the Fas-Fadd interaction, which is essential in the DISC (death inducer signalling complex) for the recruitment of pro-caspase-8 and further downstream signalling. Our results and observations from the literature, prompted us to investigate the role of Tec kinases in cell survival and its regulation by IGF-I.

2. Materials and methods

2.1. Reagents

Recombinant human (rh) IGF-I was kindly provided by Lilly Research Laboratories (Indianapolis, IN). Contamination with endotoxin was not detectable using the Limulus Amebocyte Lysate assay (detection limit 1.25 μ g/ μ g; Biowhittaker, Walkersville, MD). GM-CSF was obtained from PeproTech (London, UK). RPMI-1640 with glutamax-I, Dulbecco's modified Eagle medium (DMEM) with glutamax-I, penicillin/streptomycin (P/S) and fetal calf serum (FCS) were obtained from Invitrogen (Merelbeke, Belgium). Recombinant human transferrin, selenium, wortmannin, LY294002, dimethylsulfoxide (DMSO), blasticidin-S hydrochloride, propidium iodide (PI) and bovine serum albumin (BSA) were obtained from Sigma (Bornem, Belgium). [Methyl-³H]-thymidine and horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG were obtained from Amersham Pharmacia Biotech (Roosendaal, The Netherlands). FuGENE® 6 transfection reagent was obtained from Roche (Mannheim, Germany). Trypan blue was obtained from Merck Chemicals (Darmstadt, Germany) and the Annexin-V/FITC kit was purchased from Bender Medsystems (Vienna, Austria). Rabbit anti-Tec was from Upstate (Milton Keynes, UK). Goat anti-Btk, anti-Bmx, anti-actin and horseradish peroxidase (HRP) conjugated donkey anti-goat were obtained from Santa Cruz Biotechnology (Boechout, Belgium) while rabbit anti-Akt and rabbit anti-phospho-Akt (pS⁴⁷³) were obtained from Biosource (Nivelles, Belgium).

2.2. Cell culture

Human neutrophils were purified from heparinized venous blood drawn from healthy donors as described previously [25]. Informed consent was obtained from all blood donors and the research protocol has been approved by the local ethical committee. Freshly isolated neutrophils were suspended in serum free medium (SFM: RPMI 1640 with glutamax-I, supplemented with 0.1% BSA (A-2153),

100 U/ml penicillin and 100 μ g/ml streptomycin). BSA (A-2153) was of standard grade quality and selected by us, because it contained undetectable levels of IGF-I (<0.05 ng/mg). For the translocation experiments, neutrophils were treated in eppendorf tubes (Eppendorf AG, Hamburg Germany) in a pre-warmed (37 °C) water bath. Kinase inhibitors or vehicle (DMSO) were always applied 30 min before addition of IGF-I or GM-CSF.

Human embryonic kidney cells (Hek293T) used for all experiments were obtained from R. Beyaert (University of Ghent, Belgium). Hek293T cells were grown in DMEM containing 100 U/ml penicillin and 100 μ g/ml streptomycin (P/S) supplemented with 10% FCS in a humidified 5% CO₂ atmosphere at 37 °C. To avoid interference with growth factors and IGF binding proteins from serum, we utilized SFM to perform our experiments.

2.3. Transient transfection

Full length Tec kinase (mouse Type IV) inserted at BamHI-EcoRI of pBluescript was cut out by XbaI and EcoRI and ligated to pSSR α vector at XbaI-EcoRI (pS-Tec) [17]. The empty pSSR α plasmid (pS-Vec) was used as a negative control. A Tec kinase deleted plasmid (BSR-TecKD) and a Tec kinase mutated plasmid (BSR-TecKM) were used to study the role of the kinase domain, and Tec kinase without the SH3 domain was used as a constitutively active Tec kinase (BSR-TecSH3). The latter plasmids carry a blasticidin-S (BSR) selection gene that allowed us to recover BSR-gene expressing Hek293T cells.

For transfection with pSSR α -Tec constructs, 0.5 \times 10⁶ Hek293T cells were seeded in a 24-well plate (Greiner, Wemmel, Belgium) in 1 ml culture medium (DMEM/10% FCS without antibiotics). The cells were grown overnight to allow adherence in humidified 5% CO₂ atmosphere at 37 °C. Then, the cells were transfected with 2 μ g of pSSR α -Tec plasmid or other construct of interest using FuGENE® 6 reagent for 72 h. As a control, Hek293T cells were transfected with an empty pSSR α vector.

2.4. Stable transfection

Hek293T cells were transfected as described above with the plasmids containing the blasticidin-S Resistance (BSR) gene and harvested 72 h post-transfection (similar to transient transfection). The cells were diluted to a concentration of 1 cell/well in a 96 well plate. Next, the cells were allowed to attach for 24 h in normal culture medium before adding the selective antibiotic. Blasticidin-S (10 μ g/ml) was used to positively select the cells containing the plasmid of interest. As a control, non-transfected Hek293T cells were also grown in the selective medium. The medium was changed every 2–3 days in the first week and every 5 days during the following 2 weeks. After 2 weeks, clones were selected and expanded in 250 ml culture flasks (Cellstar, Greiner). Transfected cells from the different clones were then harvested and levels of wild type and mutated Tec kinase proteins were assessed by western blotting. Control cells that were not transfected died within one week upon exposure to 10 μ g/ml blasticidin-S.

2.5. Immunoprecipitation assay

Immunoprecipitation was performed on membrane fractions obtained from 40 \times 10⁶ neutrophils. Cells were pre-incubated for 30 min in SFM (37 °C) and subsequently stimulated for 20 s with either vehicle or 300 ng/ml IGF-I. Stimulation was stopped by addition of 900 μ l ice-cold KCl relaxation buffer and membrane fractions were isolated as previously described [1]. Subsequently, membranes were lysed in 100 μ l buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1% NP40, 2.5 mM sodium orthovanadate, 10 mM Na₄P₂O₇, 50 mM NaF, 50 μ g/ml leupeptin, 10 μ g/ml aprotinin, 50 μ g/ml soybean trypsin inhibitor, 50 mM pepstatin A and 1 mM AEBSF) for 10 min on ice. For immunoprecipitation 2 μ g of anti-Tec kinase antibody

(Upstate, cat# 06-531) or 2 μg anti-phosphotyrosine antibody (Upstate, clone 4 G10) was added to the membrane fractions. The reaction tubes were put on a vertical rotating platform for 2 h at 4 °C. Then, 20 μl of a 50% protein-G Sepharose solution (Amersham Biosciences AB) was added and reaction tubes were put on the vertical rotating platform for 1 h (4 °C). After 3 wash steps with the lysis buffer, 20 μl buffer was added followed by an equal amount of 2 \times sample buffer (2% β -mercaptoethanol, 20% glycerol and 4% SDS). The samples were boiled for 7 min. For each of the 4 conditions shown in Fig. 2D, all precipitated material was loaded onto an 8.5% gel and western blotting was performed.

2.6. Western blotting

Transient or stable transfected Hek293T cells were lysed for 10 min in ice-cold lysis buffer (double distilled (dd) H₂O with 1% NP40) containing 1 tablet of Complete protease inhibitor cocktail and 1 tablet of PhosStop phosphatase inhibitor cocktail (Roche, Mannheim, Germany). An equal amount of 2 \times sample buffer was added and samples were boiled at 100 °C for 7 min. To ensure equal amounts of protein loading in each lane (20 $\mu\text{g}/\text{lane}$), a Bradford protein assay was carried out. Proteins from Hek293T cells or membrane fractions from neutrophils were subjected to SDS-PAGE (8.5%) and then transferred to PDVF (Immobilon, Millipore) membranes. After saturation of non-specific binding sites using blocking buffer (PBS containing 5% non-fat milk/0.1% Tween 20), blots were probed with primary antibodies (1/1000 in PBS containing 5% BSA and 0.1% Tween 20) overnight for 18 h at 4 °C. The blots were then washed three times for 10 min in PBS/0.1% Tween 20 and incubated with a species-specific HRP-labelled IgG (1/5000) in blocking buffer for 1 h at room temperature. After washing for another 30 min, immunoreactive bands were revealed using the enhanced chemiluminescent (ECL) western blotting kit as recommended by the manufacturer (Amersham life Science; Diegem; Belgium). As a loading control, blots were labelled with an anti-actin antibody after stripping. First, the blot was washed for 10 min in double distilled H₂O and incubated for 30 min at room temperature in 0.25 M sodium hydroxide containing 0.1% azide. After another 10 min in dd H₂O, blots were incubated for 1 h with blocking buffer followed by incubation with anti-actin antibody (Santa Cruz Biotechnology) in 5% non-fat milk/0.1% Tween 20 overnight for 18 h at 4 °C. Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick MO).

2.7. Proliferation assay: measurement of DNA synthesis

Each condition was tested in five fold. After 24 h of culture in SFM, 1 μCi [³H] thymidine was added to each well. Cells were further incubated for 24 h, after which DNA synthesis was determined by measuring the incorporation of [³H]-thymidine. DNA was isolated using a multicell harvester (PerkinElmer, Filtermate Harvester) and radioactivity was measured by liquid scintillation counting (Wallac 1450 MicroBeta).

2.8. Cell viability assay: trypan blue exclusion test

Cell survival was monitored after 48 h of serum deprivation by the trypan blue exclusion test. Both adherent cells and cell suspensions were collected and centrifuged at 200 g for 10 min at 4 °C. The cells were resuspended in 40 μl of 0.1% trypan blue in PBS immediately before counting using a standard hemacytometer (Superior, Germany). The trypan blue exclusion test is used to determine the number of viable cells present in cell suspension.

2.9. Analysis of apoptosis: Annexin-V/PI staining assay

One of the earliest changes seen in cells undergoing apoptosis is the externalisation of the plasma membrane phospholipid

phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin-V, a phospholipid-binding protein has a high affinity for PS. Since externalization of PS occurs very early in apoptosis, Annexin-V can be used to identify apoptosis at an earlier stage than techniques based on nuclear changes such as DNA fragmentation. In this study, we used Annexin-V conjugated with FITC and propidium iodide (PI), which stains DNA, to identify necrotic cells. Transfected Hek293T cells (with the plasmid of interest, transiently or stable transfected) were collected after treatment (see Results section) and washed twice with ice-cold PBS and centrifuged at 200 g for 5 min at 4 °C. The cells were resuspended in 90 μl of Annexin-V binding buffer after which 5 μl of a 20 $\mu\text{g}/\text{ml}$ solution of propidium iodide (PI) and 5 μl of Annexin-V-FITC (Bender Medsystems, Austria) were added. After 15 min of incubation in the dark at room temperature, the samples were analyzed with a FACS Vantage flow cytometer with a 488 nm argon laser (Becton Dickinson). For each sample, forward light scatter, side scatter, FL1 (FITC; green fluorescence) and FL2 (propidium iodide; red fluorescence) of 10,000 cells were acquired. Debris was excluded by gating on basis of forward and side scatter. Data analysis was performed using WinMDI 2.8 software (URL: <http://facs.scripps.edu/software.html>).

3. Results

3.1. Translocation of Tec kinase

As previously reported [1], Fas-induced apoptosis in human neutrophils is inhibited by IGF-I, which was dependent of PI₃K but the downstream target Akt was not phosphorylated in the IGF-I signalling transduction. Because Akt was not the downstream mediator of PI₃K in IGF-I signalling, we investigated other downstream targets of PI₃K. After confirming that PKC δ was not the downstream target of PI₃K through inhibitor studies (data not shown) we assessed whether Tec kinase family members are involved in the IGF-I signalling by studying the translocation of these proteins to the membrane.

We isolated human neutrophil membrane fractions through differential centrifugation after stimulation of these cells for 20 s with SFM, IGF-I or GM-CSF. GM-CSF was chosen as a control, because Tec kinase family members are involved in the signal transduction of GM-CSF in other hematopoietic cells [14] and it is a known anti-apoptotic factor in spontaneous and Fas induced apoptosis [2,26,27]. In addition, GM-CSF also induces the translocation of Akt (also a kinase that translocates due to its PH domain) to the membrane in a PI₃K dependent fashion.

Fig. 1A shows that in response to IGF-I, only Tec kinase is translocated to the cell membrane of neutrophils. We found that 20 s was the best time point to obtain consistent results concerning the translocation of Tec kinase to the membrane fraction. The time point of 20 s was chosen for all our experiments after performing several time line experiments (data not shown). This finding is in accordance with observations of others [5] showing that translocation of Tec kinase family members to the membrane occurs within seconds. They showed that Tec kinase was translocated to the membrane within 5 s and disappeared after 1 min of incubation with fMLP. Fig. 1B shows that IGF-I significantly stimulates the translocation of Tec kinase but not of Akt to the membrane. The latter is consistent with our previous finding that Akt is not phosphorylated in response to IGF-I [1]. In contrast, stimulation of neutrophils with GM-CSF did not lead to translocation of Tec kinase or Btk to the cell membrane (Fig. 1C). However, GM-CSF increased the concentration of phosphorylated Akt at the membrane in neutrophils (Fig. 1D).

3.2. Role of PI₃K in the translocation and activation of Tec kinase

Next we assessed which role PI₃K played in the translocation to the membrane of Tec kinase and Akt in human neutrophils. To this

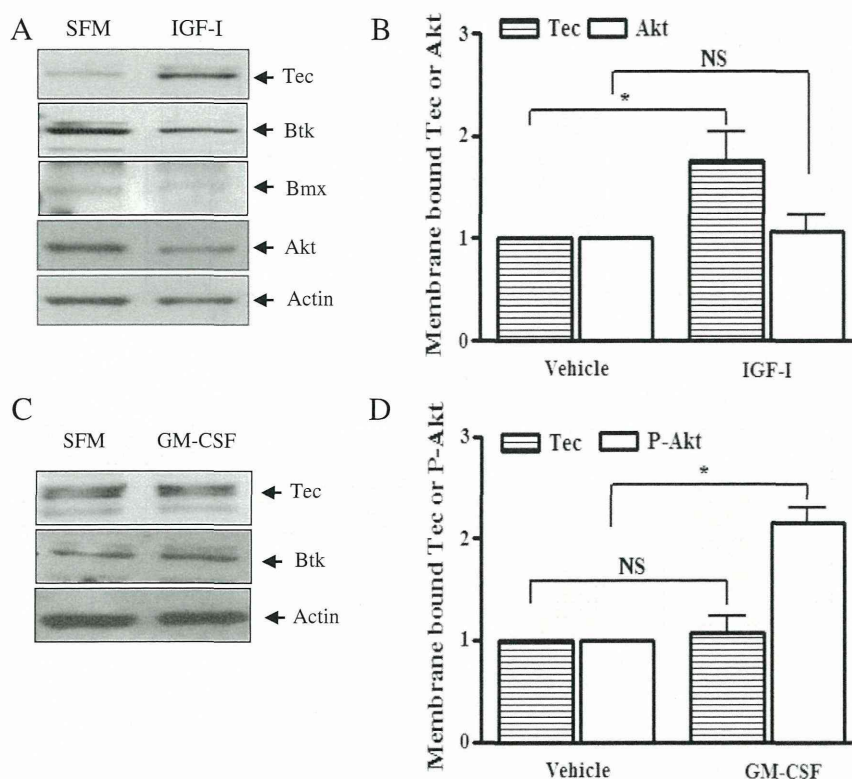


Fig. 1. Effects of IGF-I and GM-CSF on the recruitment of Tec family members and Akt to the membrane. Neutrophils were pre-incubated for 30 min in SFM and subsequently stimulated with vehicle (SFM), 300 ng/ml IGF-I (A) or 50 ng/ml GM-CSF (C) for 20 s. Western blots are representative of at least three independent experiments using different blood donors. The histogram represents the fold-change in membrane-bound Tec kinase and Akt (B) or P-Akt (D) normalized against actin. Values are mean \pm SEM from at least three independent experiments using different blood donors. Significance was determined using a one-sample *t*-test. **P*<0.05; NS (not significant).

end we pre-incubated neutrophils with vehicle (SFM + DMSO), 100 nM wortmannin (WRT) or 10 μ M Ly294002 (LY) 30 min before the cells were stimulated with IGF-I or GM-CSF. Fig. 2 (A and B) shows that the translocation of Tec kinase to the membrane by IGF-I is attenuated by WRT and LY respectively, although the PI₃K inhibitors alone also stimulate translocation to the membrane. The latter effect of WRT was not observed for Akt. Fig. 2C shows that WRT attenuates the increased amount of P-Akt at the membrane after stimulation with GM-CSF confirming the role of PI₃K in the signal transduction via Akt and the effectiveness of the inhibition by WRT.

Next, we assessed whether Tec kinase is fully activated by IGF-I. Due to the lack of specific antibodies against tyrosine phosphorylated Tec kinase we decided to perform immunoprecipitation with an anti-P-tyrosine antibody followed by immunoblotting with an antibody against Tec kinase. The left panel of Fig. 2D confirms the results found in Fig. 1A. We showed that the amount of immunoprecipitated Tec kinase from membrane fractions is almost 2.5 times increased by IGF-I (see Fig. 2E). Remarkably, when we immunoprecipitated the membrane fraction with an antibody against phospho-tyrosine (the right panel of Fig. 2D), the amount of Tec kinase in the condition stimulated with IGF-I was equal to that of the control (vehicle). Hence, the amount of tyrosine phosphorylated Tec kinase in the membrane is not increased by IGF-I.

3.3. Transient and stable transfection

Neutrophils are end-stage differentiated cells with a short half-life and low levels of mRNA expression and protein synthesis. This renders these cells difficult to transfect with constructs created to investigate protein function. Several groups already tried to transfect or transduce human neutrophils with different success rates [28–32].

Since the cellular function of Tec kinase is not well known in general, we have investigated the effects of overexpression of wild type and mutated Tec kinase molecules in Hek293T cells. To establish the functional role of Tec kinase in cell survival we transfected Hek293T cells with the empty vector (pS-Vec), two wild type Tec kinase plasmids (pS-Tec and BSR-Tec) and the Tec kinase domain deleted vector (BSR-TecKD). After 72 h, the cells were harvested and counted with the trypan blue exclusion test to evaluate the total number of living cells within the transfected populations (data not shown). We found that overexpression of Tec kinase (pS-Tec) induces a 30% (\pm 7% SEM; *n* = 3) increase in the percentage of living cells compared to cells transfected with the empty vector (pS-Vec). Similar findings were observed when the cells were transfected with the Tec kinase vector carrying the blasticidin-S resistance gene (BSR-Tec). Furthermore, we found that the pro-survival effect is completely abrogated by deletion of the kinase domain. In order to obtain long-term effects of Tec expression and to avoid the effects of the transfection procedure, we created stable transfected cell lines using several plasmids containing the blasticidin-S Resistance gene (BSR). To investigate the role of different Tec kinase domains, we used constructs with cDNA of wild type and mutated Tec kinase. We harvested each cell line (expressing a different Tec kinase), and let them adhere in a 24 well plate in 10% FCS. The following day, we removed the culture medium and rinsed the cells with SFM. After 48 h in SFM, the cells were harvested and counted with trypan blue exclusion test. Fig. 3A confirms our initial results, obtained with transient transfection, concerning the pro-survival effects of Tec kinase. Hek293T cells stably transfected with the BSR-Tec plasmid showed a significant increase in cell survival. The BSR-TecSH3 cell line, expressing the constitutively active Tec kinase, shows a further increase in the fraction of living cells. In contrast, the kinase deleted Tec kinase (BSR-TecKD) did not affect the percentage of living cells. Remarkably, the BSR-TecKM

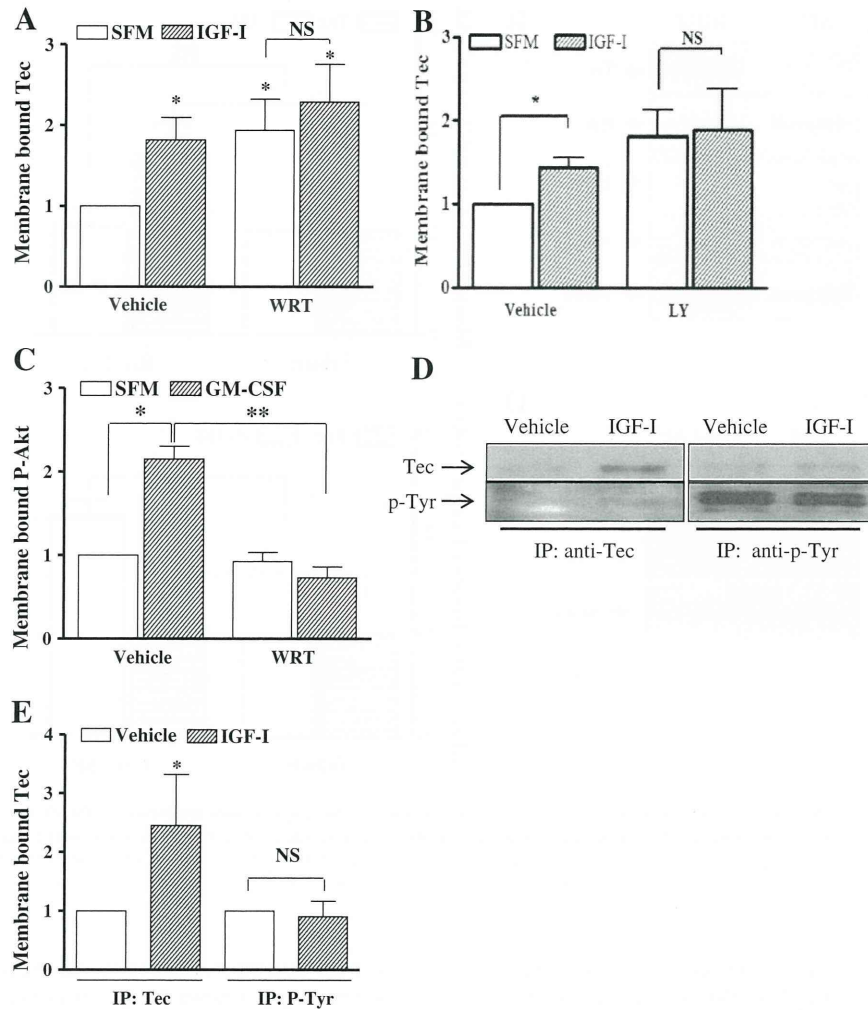


Fig. 2. Involvement of PI_3K on the translocation of Tec kinase to the membrane by IGF-I. Neutrophils were pre-incubated for 30 min with vehicle (SFM + DMSO), 100 nM WRT (A) or 10 μ M LY (B), and subsequently stimulated with SFM or 300 ng/ml IGF-I (A and B) or 50 ng/ml GM-CSF (C) for 20 s. The concentrations of DMSO in vehicle were 0.90% and 0.45% in A and B, respectively. The histogram represents the fold-change in membrane bound Tec kinase normalized against actin. Values are mean \pm SEM from ten (A) six (B) or three (C) independent experiments using different blood donors. To determine the effect of IGF-I, GM-CSF, WRT or LY versus the control (vehicle + SFM) a one-sample *t*-test was used. To determine the effect of IGF-I versus SFM in the presence of WRT or LY a student *t*-test was used. * $P < 0.05$; NS (not significant). (D) After stimulation with SFM or IGF-I for 20 s, membrane fractions were isolated and immunoprecipitation was performed with anti-Tec kinase or anti-p-Tyr antibody. Immunoblots were labelled with anti-Tec kinase (Tec) and anti-phospho-tyrosine (p-Tyr) antibody. Each lane represents immune-precipitated material from the membrane fraction of 40×10^6 neutrophils. This experiment is representative of three independent experiments using 3 different blood donors. (E) The histogram represents the fold induction of membrane-bound Tec kinase (induced by IGF-I). Values are mean \pm SEM from three independent experiments using different blood donors. To determine the effect of IGF-I versus SFM a one-sample *t*-test was used. * $P < 0.05$; NS (not significant).

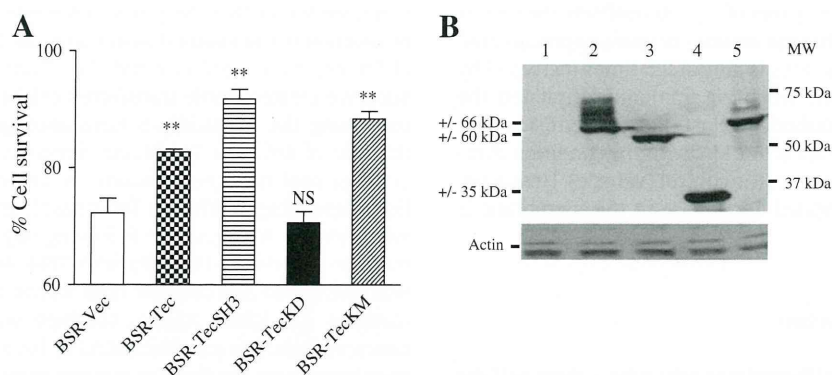


Fig. 3. Effect of stably transfected Hek293T cells on cell survival assessed by the trypan blue exclusion test (A). Values are mean \pm SEM from quadruplicate incubations and representative of three independent experiments. To determine the significance of the effect of the stably transfected cell lines versus BSR-Vec, a one-way ANOVA with a Dunnett post-test was used. * $P < 0.05$; ** $P < 0.01$; NS (not significant). (B) Representative western blot of several Tec kinase mutants with the BSR gene overexpressed in Hek293T cells (MW = molecular weight of markers). Cells were transfected with 2 μ g of BSR-Vec (1), BSR-Tec (2), BSR-TecSH3 (3), BSR-TecKD (4) or BSR-TecKM (5).

with a single amino acid mutation in the kinase domain also increased the percentage of living cells. This result suggests that the survival effect of Tec kinase is dependent on its kinase domain, but not on its kinase activity. Fig. 3B is a representative western blot showing the expression of different Tec kinase forms in Hek293T cell lines.

3.4. Tec kinase and cell survival

In order to further analyse the role of Tec kinase in cell survival we repeated the same experiment and further analysed the effect of these Tec kinase proteins at the level of apoptosis. Using Annexin-V/propidium iodide labelling in combination with the flow cytometry we determined the effect on apoptosis and necrosis. Fig. 4A shows that overexpression of Tec kinase and its constitutively active form again increases the percentage of living cells. Furthermore, the kinase dead Tec kinase reduces the fraction of living cells compared to the cells expressing the empty vector (BSR-Vec). Fig. 4B reveals that the effect of Tec kinase overexpression on cell survival is due to inhibition of necrosis, while the fraction of apoptotic cells is unaffected. Similarly, inhibition of cell survival by BSR-TecKD is mediated by stimulation of necrosis.

In order to investigate whether the increase in percentage of living cells may be due to stimulation of cell proliferation we measured DNA synthesis in transfected cell lines. We cultured stably transfected cell lines for 48 h in SFM. Fig. 4C clearly shows that neither one of the Tec kinase constructs affects DNA synthesis, suggesting that the effect on the percentage of living cells is a direct effect on cell survival.

4. Discussion

We previously showed that IGF-I plays an important role in regulating apoptosis in neutrophils. Regulation of the neutrophil apoptotic machinery is crucial for the resolution of inflammation. Deregulation of neutrophil apoptosis can be instrumental to acute or chronic diseases because delayed neutrophil apoptosis leads to a prolonged duration of inflammation. For instance, neutrophil apoptosis is delayed in the bronchoalveolar lavage (BAL) of acute respiratory distress syndrome patients (ARDS). These patients suffer from alveolar damage which is associated with increased cytokines and growth factor levels as well as reduced survival. One of these growth factors is IGF-I. Elevated levels of IGF-I and IGF-I receptors have been shown in lung biopsy samples from patients with fibroproliferative ARDS (FP-ARDS) [33]. Furthermore, when neutrophils were cultured with the BAL of ARDS patients, apoptosis in neutrophils was delayed [34]. Until now, this delayed neutrophil apoptosis was linked to other growth and inflammatory agents such as GM-CSF [27,26,35], G-CSF [36,37], IFN β [38], IFN α , IFN γ [39] and IL-8 [40–42]. So a better understanding of IGF-I signalling transduction in human neutrophils is important for the development of therapeutic drugs targeting neutrophils in acute or chronic inflammatory diseases.

We investigated the expression and the possible role of Tec family kinase members in the IGF-I signalling cascade in neutrophils. It is intriguing that many factors that inhibit neutrophil apoptosis (G-CSF, GM-CSF, IL-8 and fMLP) recruit Tec kinase members to the membrane. For instance, it was shown by Zemans and Arndt [8] that Tec kinase was recruited to the plasma membrane by LPS and that IL1 β expression was

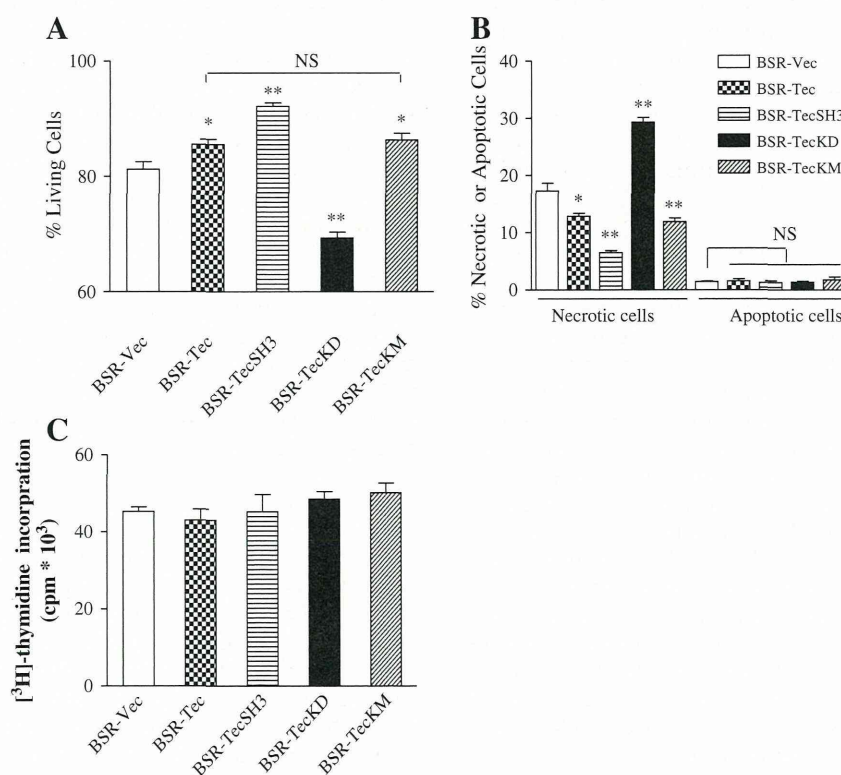


Fig. 4. Effect of stably transfected Hek293T cells on cell survival and DNA synthesis. (A–B) Each cell line was plated in a 24 well plate in 10% FCS for 24 h before the culture medium was removed and SFM was added for 48 h. The cells were harvested, labelled with Annexin-V/propidium iodide and analysed with FACS to determine the percentage of living cells (A) or necrotic and apoptotic cells (B). Values are mean \pm SEM from triplicate incubations and representative of three independent experiments. To determine the significance of wild type Tec kinase and mutated proteins versus the control, a one-way ANOVA with a Dunnett post-test was used. * $P < 0.05$; ** $P < 0.01$; NS (not significant). (C) Stable transfection of Hek293T cells with Tec kinase constructs does not affect DNA synthesis. Hek293T cells were cultured for 48 h. After 24 h, 1 μ Ci [³H]-thymidine was added. DNA synthesis was determined after 48 h of incubation period by measuring the incorporation of [³H]-thymidine. Values are mean \pm SEM from fivefold incubations and representative of three independent experiments. To determine the significance of the effect of Tec kinase constructs, a one-way ANOVA with a Dunnett post-test was used. None of the constructs significantly affected DNA synthesis.

dependent on Tec kinase activity. In addition, Tec kinase was also recruited by the chemotactic factor fMLP and after CD16b cross-linking in neutrophils. The latter two activators are playing a crucial role in the defence function of neutrophils. Two of the most important functions of these cells are recognition of bacterial components (e.g.: LPS and fMLP) or Fc regions from antibodies that opsonize antigens.

As already shown by others [5,8], we confirmed that Tec kinase, Btk and Bmx are expressed in neutrophils but we are the first to show that IGF-I stimulates Tec kinase recruitment to the plasma membrane of neutrophils. Translocation seems to happen in a similar pattern as described by Lachance et al. [5], which is very fast and short in duration. Another interesting finding is that Tec kinase is the only member of its family being recruited after stimulation with IGF-I. In contrast, Tec kinase, Bmx and Btk were all recruited after stimulation with fMLP suggesting that IGF-I has its own specific signalling transduction pathway in these cells.

After establishing that inhibition of Fas-induced apoptosis in neutrophils by IGF-I was PI₃K dependent, we showed that induction of Tec kinase recruitment to the plasma membrane by IGF-I was abrogated by wortmannin (WRT), which is in accordance with the results of Lachance et al. [5] who showed that when neutrophils were stimulated with fMLP in the presence of wortmannin, Tec kinase translocation induced by fMLP was markedly inhibited [5].

After proving that IGF-I recruits Tec kinase to the membrane fraction through PI₃K, we addressed the effect of IGF-I on the phosphorylation of Tec kinase. We show that the increased amount of Tec kinase on the membrane fraction does not lead to increased phosphorylation of the protein. Several explanations can be given for this phenomenon. It is possible that Tec kinase when phosphorylated at the membrane is immediately released to the cytoplasm. However, since we didn't find phosphorylated Tec kinase in the cytoplasm it's possible that only a small fraction of cytoplasmic Tec kinase is being recruited to the membrane. Another explanation can be that IGF-I is only responsible for the recruitment of Tec kinase but not for tyrosine phosphorylation, which would then require another factor to activate the Src kinase (involved in tyrosine phosphorylation of Tec kinase).

Several groups have already presented evidence that Tec kinase family members can exert anti-apoptotic effects. Most interestingly, Melcher et al. [12] observed an impaired survival rate and a 75% reduction in cell number in cultured Tec^{-/-}Btk^{-/-} macrophages compared to the wild-type macrophages, although the Tec^{-/-} macrophages showed a less severe reduction in cell numbers. Increased activated caspase-11 which is involved in caspase-3 activation was found not only in Tec^{-/-}Btk^{-/-} macrophages but also in Tec^{-/-} and Btk^{-/-} single knockout macrophages. Furthermore, it is to our best knowledge that there is no direct evidence for the role of Tec kinase in cell survival. However, other studies investigating the role of Tec kinase family members, suggest a role in regulating apoptosis. Vassilev et al. [24] showed that in chicken B-lymphoma cells (DT40) Btk binds to the Fas receptor where it prevents the Fas-Fadd interaction, which is essential in the DISC (death inducer signalling complex) for the recruitment of caspase-8 and further downstream signalling. Clear evidence for an anti-apoptotic role of Tec kinase family members other than Tec kinase itself is obtained by studying X-linked agammaglobulinemia [19,20], where it is believed that mutations in the Btk gene leads to an inappropriate apoptotic cell death of pre-B-cells. In addition, Honda et al. [22] showed that neutrophils from patients with XLA showed a significantly higher percentage of apoptotic cells after stimulation with TNF α and fMLP compared to normal neutrophils.

We confirmed that overexpression of Tec kinase increased the cell survival using stably transfected cell lines (Fig. 3A). BSR-TecSH3 showed a further increase of the fraction of living cells compared to BSR-Tec, reproducing the results of others. Yamashita et al. [43] presented data which showed that deletion of the SH3 domain resulted in a hyperphosphorylated and activated Tec kinase while Seidel-Dugan et al. [44] evaluated mutants of c-Src lacking its SH3 domain and also found a much higher *in vitro* level of kinase activity. It is believed that the SH3

domain of Tec kinase can bind its own proline-rich regions in the TH-domain which results in kinase inactivity. In stably transfected cells the BSR-TecKD construct reduced cell survival, possibly by acting as a dominant-negative molecule. It remains to be established which Tec kinase family member is targeted by the dominant-negative action of this molecule. An interesting finding from our experiments is that Tec kinase with a mutated kinase domain (BSR-TecKM: a kinase-dead Tec kinase due to replacement of Lysine in the ATP binding site with Methionine) did not show similar effects as the BSR-TecKD reduction in the percentage of living cells. The possibility exists that Tec kinase with a mutated kinase domain does not completely act as a dominant negative mutant.

One group already [45,46] showed that expressing a kinase inactive form of Btk in DT-40 B cell line (Btk deficient B cells that are defective in BCR-induced PLC γ 2 phosphorylation and calcium mobilization) could reconstitute calcium mobilization. The same group also described that kinase inactive Btk can reconstitute BCR-induced Erk activation. It is thought that Btk and especially Btk's PH domain-PIP3 interaction is important in the signalosome at the plasma membrane for further downstream signalling (PLC γ 2 activation) which can be independent of the kinase activity. Btk (just like Tec kinase) has an SH2 and SH3 domains where several other molecules can bind to [47]. Hence, it is possible that Btk may act partially as an adaptor molecule and function independently of its catalytic activity.

Hao et al. [48] showed that serum response factor (SRF) activation by anti-IgM was independent of the kinase domain of Itk but dependent of its SH2 domain. Furthermore, Erk activation was also independent of the kinase domain of Itk.

The possibility that Tec kinase acts as an adapter molecule and our observations that Tec kinase is translocated to the plasma membrane after stimulation with IGF-I but not phosphorylated, prompted us to hypothesize that Tec kinase acts in our experimental model rather as an adapter molecule than as a kinase. This also explains our observation that the kinase mutated Tec kinase (BSR-TecKm) didn't show a reduced percentage in living cells (Fig. 4A) compared to the kinase domain deleted Tec kinase (BSR-TecKd). We postulate that the latter can be explained by conformational changes in the 3D conformation. So it is possible that Tec kinase with a deletion of its kinase domain cannot act as an adaptor molecule due to the changed conformation. In addition, we hypothesize that Tec kinase is a potential downstream target of the IGF-I signalling pathway in neutrophils and that Tec kinase is involved in the IGF-I inhibition of Fas-induced apoptosis in neutrophils.

Further research concerning the role of IGF-I in general inflammatory diseases concerning the deregulation of neutrophil apoptosis together with further unravelling the downstream signalling pathways of IGF-I in neutrophils, may be a good strategy to find potentially new targets to develop therapeutic treatments for acute and chronic inflammatory diseases.

5. Conclusions

In summary, we previously showed that IGF-I inhibits neutrophil apoptosis via activation of PI3K, independently of its downstream target of Akt. Here, we show that IGF-I stimulates the PI3K-dependent translocation of Tec kinase without affecting its phosphorylation level. We also provide, for the first time, evidence that Tec kinase is able to stimulate cell survival using transfected Hek293T cells, and we hypothesize that in these cells Tec kinase functions as an adapter molecule independent of its kinase activity. This idea is in accordance with a possible role of Tec kinase translocation without subsequent phosphorylation in regulating neutrophil survival.

Declaration of interest

All authors have approved the final article and declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Computational dissection of distinct microRNA activity signatures associated with peripheral T cell lymphoma subtypes

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In hematological malignancies, various studies on microRNA (miRNA) profiling have highlighted specific miRNA signatures associated with several clinical conditions, such as disease subtypes, drug responses, and clinical outcomes in leukemia and B cell lymphomas, in parallel with the mRNA profiling study.^{1,2} On the other hand, the characterization of these disease-related miRNA signatures is often compromised by the poor overlapping results of multiple independent studies, even if they focused on the same disease.^{3,4} This problem may be partly explained by a poor overlap among differentially expressed miRNAs identified by multiple miRNA microarray platforms.⁴ One promising approach is the paired profiling of mRNAs and miRNAs, and integrated analysis of both to extract disease-related features. Although the pairing of miRNAs and target mRNAs can be inferred from anticorrelation between miRNAs and mRNAs, the generally weak impacts of miRNAs on mRNA expression levels still make this approach difficult.

To this end, we have recently developed GSEA–FAME analysis (GFA) through combining GSEA and FAME to consider weak mRNA changes and the variability of the strength of correlations between an miRNA and its target genes.⁵ GFA utilizes mRNA expression profiling to predict alterations in miRNA activities through rank-based enrichment analysis and evaluation of weighted miRNA–mRNA interactions (Figure 1a).⁵ We demonstrated that GFA is useful for the assessment of widespread correlations between miRNA expression levels and miRNA activity status in diffuse large B cell lymphoma, and that GFA-based inference of miRNA activity improves the extraction of prognostic miRNAs by miRNA profiling in TCGA glioblastoma data set.⁵ In this report, we applied this analysis to the gene expression profiling of peripheral T cell lymphomas (PTCL), in which the roles of miRNAs have not been well investigated, and made a prediction map of differential miRNA activities.

Recent miRNA profiling in anaplastic large cell lymphoma (ALCL) showed dysregulated miRNA profiles, including miR-17–92 cluster, miR-106a and miR-155, between anaplastic lymphoma kinase (ALK)+ and ALK– ALCLs.⁶ In addition, we and another group reported miR-135b upregulation and miR-29a

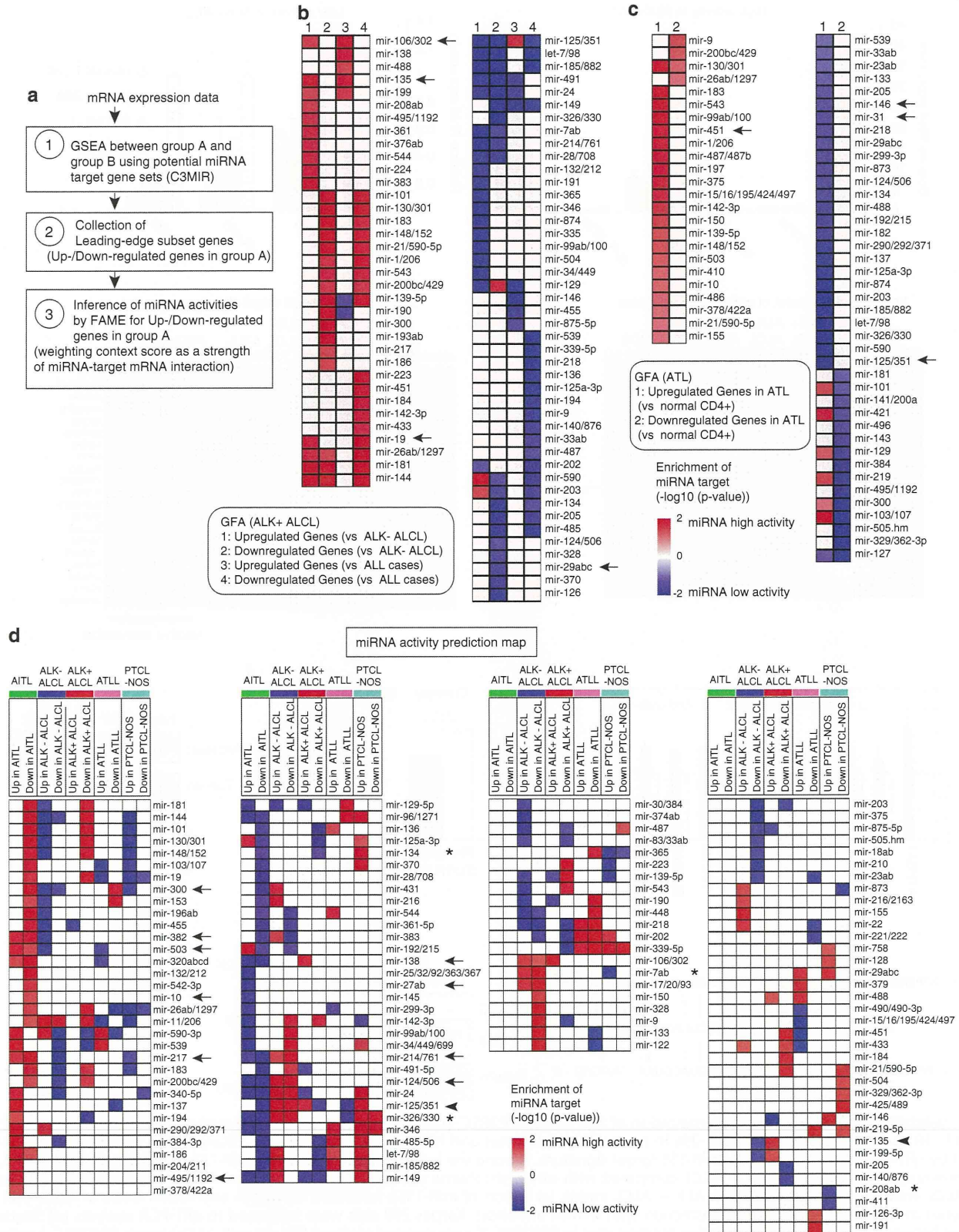


Figure 1. Prediction of miRNA activities in peripheral T cell lymphoma by GFA. **(a)** Outline of GFA. The details of GFA have been described in Supplementary Information. **(b)** GFA in ALK+ ALCL. GFA was performed between ALK+ and ALK- ALCL, and between ALK+ ALCL and all cases of PTCL in the gene expression profile of T cell lymphoma (GSE19069). miRNAs with $P > 0.05$ are shown as white squares. miRNAs indicated by arrows have been discussed in the main text. **(c)** GFA in ATL. GFA was performed between ATL cells and CD4-positive normal T cells in the gene expression profile of ATL (GSE33615). miRNAs with $P > 0.1$ are shown as white squares. miRNAs indicated by arrows have been discussed in the main text. **(d)** GFA in PTCL subtypes. For four major T cell lymphoma subtypes (AITL, ALK+ ALCL, ATLL and PTCL-NOS), GFA was performed between each lymphoma subtype and all cases of PTCL in GSE19069 data set. miRNAs with $P > 0.05$ are shown as white squares. miRNAs indicated by arrows (for AITL), arrowheads (for ALK+ ALCL and ATLL) and asterisks (for PTCL-NOS) have been discussed in the main text.