Tumor Necrosis Factor α (TNF-α) Activates Jak1/Stat3-Stat5B Signaling through TNFR-1 in Human B Cells

Sebastiano Miscia, Marco Marchisio, Alfredo Grilli, Valentina Di Valerio, Lucia Centurione, Giuseppe Sabatino, Francesco Garaci, Giorgio Zauli, Ezio Bonvini, and Angela Di Baldassarre

Cell Signaling Unit at the Department of Biomorphology [S. M., M. M., A. G., V. D. V., L. C., A. D. B.] and Section of Neonatology [G. S.], School of Medicine, University of Chieti, 66100 Chieti, Italy; Dipartimento di diagnostica per immagini e Radiologia Interventistica, University of Roma Tor Vergata, D0100 Rome, Italy [F. G.]; Section of Human Anatomy at the Department of Morphology and Embryology, University of Ferrara, 44100 Ferrara, Italy [M. M.]; Department of Human Morphology, School of Medicine, University of Trieste, 34127 Trieste, Italy [G. Z.]; and Laboratory of Immunobiology, Center for Biologistics Evaluation and Research, Bethesda, Maryland 20814 [E. B.]

Abstract
The biological actions of tumor necrosis factor α (TNF-α) are mediated by two cell surface receptors, TNFR-1 and TNFR-2. These receptors do not display protein tyrosine kinase activity. Nevertheless, an early TNF-induced activation of specific tyrosine kinases has been reported as an important cue to the cellular response to this cytokine. Here we present evidence that TNF-α induces the activation of the cytoplasmic Janus tyrosine kinases Jak1 and Tyk2 in both human healthy peripheral and lymphoma B cells. This event was accompanied by the recruitment of a specific set of latent cytosolic transcription factors, Stat3 and Stat5b. Furthermore, Jak1 coprecipitated with TNFR-1 after TNF-α treatment. These data suggest that at least in human B cells this cytokine can exert its biological effects through the Jak-Stat signaling pathway and that such signals are initiated through an interaction between TNFR-1 and Jak 1.

Introduction
TNF-α3 is an endogenous mediator of inflammatory and immune responses able to regulate cell metabolism by modulating gene expression and by controlling cell proliferation and survival (1, 2). Important progress has been made in defining the signaling intermediates of TNF activity. The intracellular signal is initiated by the binding of the cytokine to one of two specific and ubiquitously expressed receptors: TNFR-1 (CD 120 a, M, 55,000) and TNFR-2 (CD 120 b, M, 75,000). These molecules share homology in their extracellular domains but are dissimilar in their intracellular portion (3, 4). They mediate a distinct but, at least in part, overlapping repertoire of responses. TNFR-1 appears to mediate the majority of TNF actions including cytotoxicity, fibroblast proliferation, and immunological and inflammatory responses (5, 6), whereas an independent role in the proliferation of monocytes and T lymphocytes, and a function complementary to TNFR-1 in cytotoxicity have been suggested for TNFR-2 (7–9). Because the TNF receptors are devoid of enzymatic activity (3, 4), the signal appears to be mediated by the recruitment of cytosolic proteins through specific protein-protein interaction domains. Cytokine-induced receptor aggregation enables the interaction of TNFR-1 with the TNF-receptor-associated death domain protein, a protein that couples TNFR-1 to gene modulation through NFκB activation, or cell death, through the involvement of caspases (10). Ligation of TNFR-2 can affect the expression of genes involved in immediate early prosurvival responses (11), and this is also dependent on the activation of NFκB. The TNFR-2 associated factor TRAF-2 is in fact engaged in a number of protein-protein interactions that lead to the release of NFκB from its the cytosolic inhibitor, allowing its nuclear translocation (12, 13). Whereas several steps of TNF signaling have been elucidated, many of the molecular events evoked by this cytokine are still unclear. For instance, an early TNF-induced activation of tyrosine kinase activity has been reported, although neither TNF receptor displays intrinsic protein tyrosine kinase activity (3, 4). Furthermore, tyrosine kinases inhibitors suppress TNF-induced phosphorylation of a M, 23,000 nucleic protein (14), activation of NFκB (15), and DNA fragmentation (16). Additionally, recent evidence in murine adipocytes have provided additional clues to the existence of a tyrosine kinase signaling pathway involved in certain cellular response to this cytokine (17). The aim of the present study was to investigate, in human B primary and lymphoma cells, the involvement of a family of cytoplasmic protein tyrosine kinases, the Jaks, in signal transduction mediated by TNF.

Results
To characterize the susceptibility of primary B and Ramos cells to the TNF-α action, we determined effects on cell viability by trypan blue dye exclusion and on morphology by means of semithin sections after scalar doses of TNF-α (50, 100, 250, and 500 nM). Table 1 shows that after 16 h of treatment with 250 nM TNF-α, the most effective concentration in our hands, > 50% of lymphoma and primary B cells failed to exclude trypan blue. The semithin section samples disclosed the well-known apoptotic morphological changes that are hallmarks of apoptosis (Fig. 1). To shed light on the
upstream molecular events related to the biological effects exerted by TNF-α on these cells, we next investigated the possible involvement of Jak proteins. This was first accomplished by an in vivo labeling of cells with [32P]Pi, followed by TNF-α treatment and immunoprecipitation of each Jak protein with specific antisera. The administration of the cytokine induced the phosphorylation of Jak1 and Tyk2 as early as 5 min in both Ramos (Fig. 2, A and B) and primary B cells (Fig. 2, C and D). The fact that Jak2 and Jak3 phosphorylation were scarcely or not detected in either cell only influenced the number of viable cells without producing substantial changes in induced Jak1 and Tyk2 phosphorylation (data not shown). In Ramos cells we detected a M₉₀,000/60,000 phosphorylated protein coprecipitated with phosphorylated Jak1 (but not with Tyk2). We therefore reasoned that this protein could be one of the TNF receptors, namely TNFR-1, which could have recruited Jak 1. Therefore we reacted the same blot with an anti-TNFR-1 antibody. This demonstrated that the M₉₀,000/60,000 protein was indeed TNFR-1 (Fig. 2A). This result was additionally confirmed by immunoprecipitating both TNFR-1 and TNFR-2 receptors from [32P]-labeled Ramos cells treated with TNF-α. After separation of the immunoprecipitates through SDS-PAGE and transfer onto nitrocellulose and immunoblotting, autoradiography demonstrated that Jak1 was phosphorylated and associated with TNFR-1, whereas TNFR-2 was evidently phosphorylated but not associated with any phosphoprotein (Fig. 3, A and B). Similar experiments performed on primary B cells demonstrated that TNFR-1 coprecipitated with Jak1 after TNF-α treatment (Fig. 3 C), whereas TNFR-2 was neither phosphorylated nor associated with any phosphoprotein (data not shown). To assess whether Jak1 and Tyk2 were activated by TNF-α in both cell types we immunoprecipitated Jak1 and Tyk2 from control and TNF-α-treated Ramos and primary B cells, and measured their tyrosine kinase activity by a kinase assay. As shown (Fig. 4, A–D), the activities of both kinases were strongly stimulated by TNF-α.

The activation of Jak proteins is known to lead to tyrosine phosphorylation of Stat proteins. Therefore, we screened for possible Stat(s) involvement downstream of the TNF-α-mediated activation of Jak1 and Tyk2. Stat proteins were, therefore, immunoprecipitated from lysates from untreated and TNF-α-treated Ramos cells and, after separation and

<table>
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<th>Treatment</th>
<th>% of viable primary B cells</th>
<th>% of viable Ramos cells</th>
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<tr>
<td>Control</td>
<td>96 ± 2.0</td>
<td>94 ± 3.0</td>
</tr>
<tr>
<td>TNF-α (50 nM)</td>
<td>59 ± 2.5</td>
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<td>TNF-α (100 nM)</td>
<td>52 ± 3.0</td>
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<tr>
<td>TNF-α (250 nM)</td>
<td>48 ± 4.2</td>
<td>43 ± 5.0</td>
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<tr>
<td>TNF-α (500 nM)</td>
<td>49 ± 3.5</td>
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* Data are the mean of five separate experiments ± SD.
transfer onto nitrocellulose, their phosphorylation level was determined by anti-P-Tyr antibody. Only phosphorylation of Stat3 and Stat5b was evident after TNF-α/H9251 stimulation, and this was detectable within 5 min of treatment, consistent with the activation kinetics of Jak1 and Tyk2, and was clearly evident up to 40–60 min of TNF-α/H9251 stimulation (Fig. 5A). The expression and/or the phosphorylation of other Stats were either undetected (Stat5a) or not modified when compared with untreated cells (Fig. 5B). Stat3 and Stat5b tyrosine phosphorylation was also evident in primary B cells after TNF-α treatment (Fig. 5C). To investigate whether the phosphorylated Stats underwent to translocation from the cytoplasm to the nuclear compartment, we performed immunofluorescence analyses for both Stat3 and Stat5b at different times after TNF-α treatment. Both Stat3 and Stat5b translocated into the nucleus within 20 min of treatment, and this was clearly evident at 40 min of TNF-α administration (Fig. 6).

Discussion

Jaks are cytoplasmic protein tyrosine kinases that mediate cytokine signaling by phosphorylating different intracellular
targets (18, 19). In mammals, four Jak family members have been described: Jak1, Jak2, Jak3, and Tyk2. These kinases are unique in containing tandem kinase and kinase-like domains (20, 21). Their activation is promoted by the dimerization of cytokine receptors on ligand binding. Because previous studies demonstrated induction of a tyrosine kinase activity in response to TNF-α and because TNF-α receptors are devoid of known enzymatic activity, we investigated the possible involvement of Jaks in TNF-α signaling. The ability of this cytokine to induce cell death in lymphoma cells has been already described (22, 23), but thus far little information is available on the signaling pathway(s) that lead to tyrosine phosphorylation. Our data demonstrate that the administration of TNF-α to B human cells induces tyrosine phosphorylation and activation of the Jaks Jak1 and Tyk2. It is well known that binding of TNF-α, active as homotrimer, results in the clustering of monomeric TNF receptors (24, 25). This self-association seems to fit with the model suggested for Jak activation (19), allowing the juxtaposition and the transphosphorylation of the kinases. Because TNFR-1 forms homotrimers by associating with itself and with cytoplasmic proteins implicated in cell death, it is conceivable that only

Fig. 5. A–C, effect of TNF-α (5 min) on Stat phosphorylation in Ramos (A and B), and primary B cells (C). Cells were treated with the cytokine, and the proteins were immunoprecipitated from cell lysates, separated, and transferred onto nitrocellulose as described in “Materials and Methods.” Membranes were incubated with an anti-P-tyr antibody, and the same membranes were reprobed respectively with the corresponding anti-Stat antibodies to show the equal loading for each experiment. The results are representative of three independent experiments.

Fig. 6. Immunofluorescence analysis of Stat3 and Stat5b subcellular distribution after TNF-α treatment (20 min) of Ramos lymphoma cells. In regard to controls, TNF-α clearly induces the translocation of Stat3 and Stat5b into the nuclei of which the counterstaining was obtained by means of 4’,6-diamidino-2-phenylindole. Results are representative of three separate experiments.
one member of the Jak family (i.e., Jak1) is recruited. In this context, preliminary results of in vitro binding assays indicate an interaction between Jak1 and TNF-α, yet this observation needs to be additionally explored, because in the experiments reported in the present paper, the association between Jak1 and TNF-1 appears to be ligand dependent. Regardless, the finding that both Jak1 and Tyk2 become tyrosine phosphorylated and activated suggests that a Jak1/Tyk2 complex is also recruited in the TNF-1 complex. Given the well-established apoptotic role of TNF-1, the recruitment of Jak1 and Tyk2, which are generally involved in cell proliferation or survival, in the TNF-1 complex appears to be a paradoxical event, which needs more investigation. In this context it is worth noting that Jak1 and/or Tyk2 have been demonstrated recently to be involved in the induction of either apoptosis or cell growth suppression (26, 27).

These findings would argue for different roles of these Jaks that could depend on the function of either Jak homology domains or of Jak NH2-terminal region that ultimately influence the interaction of the kinase to its cognate cytokine receptor subunit. How TNFR-2 engagement may play a role remains to be determined, because in Ramos cells this receptor appears to be phosphorylated but is associated with any phosphoprotein, and in primary B cells TNFR-2 is apparently not involved in TNF signaling. These findings are more in line with the hypothesis that TNFR-2 is rather essential in TNF-dependently induced apoptosis or cell growth suppression (26, 27).

Activated Jaks phosphorylate members of a family of transcription factors, the Stats. In mammalian species, seven Stat proteins are known: Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6 (18, 19, 28-30). Tyrosine phosphorylation of the receptor by Jaks provide docking sites for Stats. Once phosphorylated, Stats are released from the receptor, dimerize, and translocate into the nucleus where they specifically bind DNA and activate the transcription of target genes, many of which are relevant for the inflammatory responses (18, 19, 31, 32). Different ligands act on a restricted sets of Jaks and Stats. Our data demonstrate that in human B cells, the TNF-α-induced Jak1 and Tyk2 activation are accompanied by Stat3 and Stat5b tyrosine phosphorylation and translocation into the nucleus, strongly demonstrating that in these cells, additional elements can control the transcriptional machinery elicited by TNF-α. How Stat3 and Stat5b activation modulates the transcriptional response of these human cells and the precise role of these factors in the cellular response to TNF-α are currently under investigation in our laboratories. Nevertheless, our observations suggest that among the different cellular responses evoked by TNF-α in B lymphoid cells, recruitment of Jak1 through TNF-1 and gene modulation by Stat3 and Stat5b may represent contributing factors.

Materials and Methods

Cell Culture and Treatment. B lymphocytes were obtained from peripheral blood of healthy consenting donors by using magnetic beads coated with anti-CD 19 monoclonal antibody (Dynabeads M-450 CD19; Dynal, Oslo, Norway). Only samples exceeding a purity of 95% were used for the experiments. Blood peripheral B lymphocytes and Ramos B lymphoma cells were grown in RPMI 1640 (Mascia Brunelli, Milan, Italy) containing 10% FCS (Mascia Brunelli), 2 mM L-glutamine, 5 mM HEPES (pH 7.3), and penicillin-streptomycin (50 IU/ml and 50 mg/ml, respectively) at 37°C with 5% CO2. The susceptibility of cells to TNF-α was monitored by determining effects on cell viability and morphology by trypan blue exclusion test and electron microscopy analysis, respectively, after incubating cells with scalar doses of TNF-α (Boehringer Mannheim, Mannheim, Germany) for 16 h. Once assessed, 250 nM was determined as the most effective dose, and molecular changes were investigated within a time interval ranging from 5 min to 60 min.

Transmission Electron Microscopy. For semithin sections, cells were fixed in suspension for 30 min with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), postfixed in 1% OsO4, and embedded in Spurr resin.

Immunocytochemical Detection of Stat3 and Stat5b. Cells were first spun onto coverslips fixed in 4% paraformaldehyde/PBS for 10 min at room temperature, washed twice with PBS, incubated with PBS for additional 15 min to quench the remaining paraformaldehyde, and saturated/permeabilized using NET gel solution [150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.4), 0.05% NP40, 0.25% Carageenan Lambda gelatin, and 0.02% Na azide] for 30 min at room temperature. After two washes with Net gel solution, cells were treated with anti-Stat3 or Stat5b rabbit polyclonal antibodies (diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at room temperature. After two washes with Net gel solution, goat antirabbit IgG (GAR-FITC, diluted 1:50) was added to the cells and incubated for 45 min at room temperature. After three additional washes (two in Net gel solution and one in PBS), the nuclei were counterstained with 4',6-diamidino-2-phenylindole (Sigma Chemical Co.) and mounted in DABCO (Sigma Chemical Co.)-glycerol-PBS. The immunostaining specificity for the monitored proteins was confirmed by the absence of any reactivity when: (a) primary antibodies directed against the other Stat proteins (diluted 1:50; Santa Cruz Biotechnology) and (b) normal rabbit serum (diluted 1:50) were used.

32P-Labeling, Solubilization of Proteins, Immunoprecipitation, and Immunoblotting. Cells were maintained for 3 h in phosphate-free DMEM, labeled with [32P]Pi (100 μCi/ml; Amersham, Milan, Italy) for 1 h, and stimulated as described above. Cells were harvested by centrifugation at 4°C, solubilized in lysis buffer [60 mM Tris-HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2 mM Na2VO4, 25 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aproxin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM TLCK (N-tosyl-L-phenylalanine chloromethyl ketone)] and normalized at 400 μg protein. Whole cell lysates were incubated at 4°C for 1 h with 1 μg of either rabbit polyclonal anti-Jak, anti-Stat, anti-TNF-1, or anti-TNF-2 antibodies (all from Santa Cruz Biotechnology) that were coupled previously to goat antirabbit IgG magnetic beads (DynaL). Immunocomplexes were collected by a magnet, electrophoresed, and transferred onto nitrocellulose. The phosphorylation level of the different proteins was assessed by autoradiography, whereas the specific tyrosine phosphorylation was assessed by
means of a mouse monoclonal anti-P-Tyr antibody (PY-99; Santa Cruz Biotechnology). The detection and the equal loading of TNFR-1 or TNFR-2 was assessed by Western blot experiments reacting the same membranes with mouse monoclonal anti-TNF-R1 and anti-TNF-R2 antibodies.

**In Vitro Kinase Assay.** Jak1 and Tyk2 were immunoprecipitated as described above from equal numbers of untreated and TNF-treated cells (2 × 10⁷). Immunoprecipitates were washed once with lysis buffer, once with 0.5 M LiCl and 20 mM Tris (pH 7.5), and once with distilled water. Samples were resuspended in 30 μl of 20 mM Tris (pH 7.5), 10 mM MnCl, and 10 μl of [γ-32P]-ATP (Amersham) per kinase assay, and incubated at room temperature for 15 min. Samples were then washed with distilled water, and proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and analyzed by autoradiography. Equal loading of samples was assessed by immunoblotting the same membranes with anti-Jak1 and Tyk2 antibodies.

**References**


