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Differential effects of tumor necrosis factor-α and CD40L on NF-κB inhibitory proteins IκBα, β and ε and on the induction of the Jun amino-terminal kinase pathway in Ramos Burkitt lymphoma cells

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ABSTRACT. Interaction between the CD40 ligand and its cognate receptor is known to affect various aspects of B-cell biology. Less is known about the biological consequences of B-cell signaling through tumor necrosis factor alpha (TNF-α) and its two receptors. We have used Ramos germinal center (GC)-derived Burkitt's lymphoma (BL) cells as a model system to compare some of the early signaling events of TNF-α and CD40L on the NF-κB and c-Jun amino-terminal kinase (JNK) pathways. We have previously found that both TNF-α and CD40L induced enhanced cell aggregation, adherence and modified cell surface morphology of Ramos cells. In the present report, it was found that treatment with either TNF-α or CD40L resulted in a rapid degradation (within 15 min) of IκBα, followed by a recovery period lasting up to a few hours. The level of IκBβ, another inhibitory molecule of the NF-κB pathway, also decreased following treatment with CD40L or TNF-α. However, whereas CD40L induced a rapid drop without significant recovery within 2 h, TNF-α caused a slow and gradual decline of IκBβ. In addition, treatment with CD40L resulted in a gradual and modest decline of up to 60% of the level of IκBε within 2 h, whereas a much smaller decline was seen with TNF-α (approx. 20%) Our results thus show that in Ramos cells, TNF-α and CD40L have common, as well as differential, signaling effects on the IκBα, IκBβ and IκBε, which form inhibitory complex(es) with the NF-κB cytosolic proteins. We also found that CD40L, but not TNF-α activates the JNK pathway through transient phosphorylation of its threonine183/tyrosine185 residues. As expected, c-Jun, which is known to be a substrate of JNK, was also phosphorylated at serine residue 73 by treatment with CD40L, but not by TNF-α.

Keywords: TNF-α, CD40L, IkB proteins, JNK, signal transduction, B lymphoma

TNF-α and CD40L are members of the large TNF family of ligands which affect various aspects of the innate and humoral immunity [1-3]. Mutations in the CD40L or its cognate receptor were found to result in hyper IgM syndrome [4]. At the cellular level, interaction between CD40L on T cells and the CD40 receptor on B cells is critical for Ig class switch recombination (CSR) and somatic hypermutation (SHM) [2, 5], which are processes that occur in the microenvironment of the germinal center (GC) [6]. Previous studies have demonstrated the role of TNF-α and its two receptors (*i.e.,* TNFR1 and TNFR2) in various aspects of B cell development [7-9] and biology [10-13]. However, it is not known whether TNF-α can, either by itself or in combination with other stimuli, directly induce B cells to generate CSR and/or SHM.

Both CSR and SHM were shown to occur in certain malignant human BL cell lines that are considered to originate from normal GC-B lymphocytes, and these cell lines were used extensively to study the nature and mechanisms of CSR [5, 14] and SHM [5, 15, 16]. One of these cell lines is Ramos, which constitutively introduces somatic mutations in its IgV-region genes [15]; however, the mutation rate seems to be about 10-fold lower than the normal rate and the cells display an abnormal preference for G/C versus A/T mutations [17]. In contrast, BL2 cells generate somatic mutations following induction by cross-linking of its B cell receptors [16]. Neither cell line displays CSR at an appreciable rate, although another BL cell line (*i.e.,* CL-01) can be induced to generate CSR and SHM [5]. In certain normal and malignant B cells, CD40L can activate a variety of signaling pathways, among which are the NF-κB and the Jun amino-terminal kinase (JNK) [18-20]. However, the details of the particular pathways involved and the mechanisms of induction of CSR and SHM are not well understood. Similarly, TNF-α is known to induce multiple cell signaling pathways in different, non-B cell types, some of which overlap with those induced by CD40L in B cells [1]. The most intensively studied NF-κB pathway includes multiple NF-κB/Rel transcription factors: Rel A (p65), Rel B, c-Rel, p50/p105(NF-κB1) and p52/p100(NF-κB2), which play a critical role in the development of certain epithelial tissues and in the response of tissues and cell types to a variety of external stimuli. Following cell triggering, these NF-κB-related transcription factors translocate as hetero/homo dimers from the cytosol into the nucleus and activate or repress, respectively, a multitude of target genes which may affect a variety of functions including cell proliferation, differentiation, viability and apoptosis [21-24]. In various cell types, the cytosolic localization of the NF-κB proteins is maintained by a group of at least eight inhibitory proteins (IκBs) consisting of the three 'classical' IκBs (IκBα, IκBβ and IκBε) the two NF-κB precursor proteins (p100 and p105) and the 'unusual' IκBs (Bcl-3, IκBζ and $I\kappa B_{NS}$) [24], which form complexes with the NF-κB factors in unstimulated cells. NF-κB activation is mediated by a complex comprising two kinases (IKKα and IKKβ) and a regulatory scaffold protein designated NEMO (i.e., NF-κB essential modulator) or IKKγ. Stimulation by various ligands activates the kinases which phosphorylate the classical κBs (IκBα, β, ε), leading to their ubiquitination and degradation via the proteasome pathway. This in turn results in the release of one or more of the NF-κB dimers and allows them to move to the nucleus [24]. Tissue-specific and ligand-specific responses of the NF-κB system were shown to depend on the expression of NF-κB transcription factors [25] and their IκB proteins [26, 27]. The NF-κB system plays a particularly important role in innate and adaptive immunity, as exemplified by gene targeting and transgenic murine experimental model systems of both NF-κB [28] and IkB genes [29-31], as well as in a group of rare, human, genetic immunodeficiency disorders associated with mutations in the *NEMO* gene [32]. Different combinations of homo/hetero dimers of the NF-κB proteins were found to be expressed during B cell maturation and activation [25, 33], and certain ligands, such as LPS, BAFF and CD40L, are able to induce NF-κB in resting B cells [28] and in human B cell lines [34]. Both quantitative and qualitative effects on cell viability have been documented for the NF-κB system [31]. There is evidence for preferential associations between certain IκB molecules and specific NF-κB dimers [35] which determine, depending on the inducing ligand, at least two types of NF-κB responses in resting B cells [28]. Type 1 NF-κB1 response to LPS, which results in enhancement of cell survival, proliferation and isotype switching, is based on the classical NF-κB activation pathway as described above, and type 2, induced by the BAFF ligand, is based on the non-classical/alternative pathway which involves proteolytic cleavage and processing of the p100 inhibitory precursor molecule and formation of its transcriptionally active p52 protein product [36, 37]. In addition to activation of an overlapping set of genes, each of these pathways also activates a distinct set of genes [28, 37-39]. Interestingly, CD40L activates both beyond the sum of the biological outcomes induced in the two types of the NF-κB responses, and it also induces B cell adhesion and homotypic aggregation [28]. However, the details of the B cell stage-specific expression of the IκB proteins and their role in regulating B cell maturation in general and the activity of GC-B cells in particular are not well understood.

In a previous study, we found that both TNF-α and CD40L affect certain biological parameters of Ramos Burkitt's lymphoma cells, such as the pattern of cell aggregation, enhancement of cell adherence and increased membrane ruffling and filopodia formation [40]. In the present report, we used Ramos cells to explore some of the initial events in the activation of the NF-κB and JNK pathways, following signaling by TNF-α and CD40L. It was found that both cytokines induced rapid degradation of the IκBα with a recovery period of a few hours. However, differential effects of TNF-α and CD40L were found on the kinetic patterns of the IκBβ and IκBε, indicating that the levels of the three IκB proteins examined are differentially regulated. In addition, CD40L, but not TNF-α, was found to activate the c-Jun N-terminal kinase (JNK) pathway in Ramos cells.

METHODS

Cell lines

Ramos and BL2 are Burkitt's lymphoma (BL) cell lines (ATCC). Two clones of Ramos were used: R1 (IgM+) and R7 (IgM-) cells which contain a stop codon in the mu heavy chain [17]. Ramos and BL2 were maintained in Iscov modified Dulbecco's medium (IMDM) supplemented with 10% FCS, 0.2 mM glutamine, 100 u/mL penicillin and 100 µg/mL streptomycin or with RPMI 1640 with 20% FCS supplemented as above.

Reagents

Recombinant human TNF-α (Sigma, Saint Louis, MO, USA) was used at a concentration of 10 ng/mL. Recombinant human trimeric CD40L (CD40LT) was a gift from Immunex Corp. (Seattle, WA, USA) and was used at a final concentration of 270 ng/mL.

Primary antibodies used for western blots were as follows: rabbit antibodies to IκBα (sc-847), IκBβ (sc-945), IκBε (sc-7155), and to p65 (sc-372) as well as mouse anti-phosphorylated c-Jun (S63) (sc-822) and mouse anti-α tubulin (sc-8035), were from Santa Cruz Biotechnologies. Mouse anti-phosphorylated SAPK/JNK (Cell signaling, #9255) and rabbit anti-phosphorylated c-Jun (S73) (Upstate cat. #06659), were used at dilutions of 1:2000 and 1:1000, respectively. HRP-conjugated sheep anti-mouse and donkey anti-rabbit IgG (Amersham, Piscatway, NJ, USA), or goat anti-rabbit IgG (H+L) HRP (Jackson Lab.) were used as secondary antibodies at dilutions of 1:1000-1:2000. Cell lysates from untreated human HL60 and THP-1 cells and from murine NIH-3T3 cells, before and after induction by UV or following heat shock, were from Santa Cruz and served as antibody specificity controls.

Cell induction by cytokines

Ramos cells (10^5cells/mL) were induced by TNF-α or CD40L in 1 mL of IMDM supplemented with 10% FCS, glutamine and antibiotics as above, in a 24-well plate, or in 200 µL of the same medium, in a 96-well plate. The cells were observed by light microscopy, and processed for western blotting at different time points up to several hours post-induction by the cytokines.

Western blot analysis

B-lymphoma cells were washed in IMDM without serum and lysed in "loading buffer" (2% SDS, 50 mM Tris-HCl pH 6.8, 10% glycerol, 0.1% bromophenol blue, 0.1M dithiothreitol (DTT) at a ratio of 100 μL of loading buffer per 10^6 cells, boiled for 5 min and 10-15 μL of each of the cell lysates were subjected to SDS-12% polyacrylamide gel electrophoresis using pre-cast ready gels (BIO-RAD Lab. Hercules, CA, USA). Following blotting, the Hybond-C-extra membranes (Amersham, Piscataway, NJ, USA) were blocked with 5% dried milk in PBS containing 0.1% Tween 20, probed sequentially with various primary antibodies overnight at 4° C, then washed, probed with anti-mouse or anti-rabbit Ig secondary antibodies coupled to HRP (Amersham, Piscataway, NJ, USA) for 2 h. at 20^0C, developed by ECL (Super Signal-Pico, Pierce, Rockford, IL, USA) and exposed to X-ray films. Stripping was performed with Restore^{TM} stripping buffer (Pierce). Mouse anti-α-tubulin was used to normalize the amount of protein per lane. For quantification, the films were scanned, and the optical density of the bands was corrected for those of the α–tubulin and normalized for the untreated control cells.

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytosolic extracts of 5x10^6 untreated or TNF-α induced (10 ng/mL, 15 min) were prepared [41] and the amount of protein in the extracts was determined by the Bradford method (Sigma, Kit). EMSA was performed essentially as described [42]. Briefly, the NF-κB binding site oligonucleotide 5'-GATCAAACAGG GGGCTTTCCCTCCTC-3', derived from the κB3 site of TNF-α promoter, or a point-mutant variant that does not bind to NF-κB (5'-AGTTGAGGCGACTTTCCCAGGC-3') were labeled with [α^{32}-P] CTP and Klenow polymerase. Nuclear and cytosolic extracts (30 μg/sample) were incubated with 30-50x10^3 cpm of the labeled probe in 20 μL of reaction buffer containing 10 mM Tris pH 7.5, 20 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 2 μg poly(dI-dC) and 4% glycerol for 20 min at room temperature. The samples were then separated on non-denaturing 7% polyacrylamide gels, which were dried and autoradiographed.

RESULTS

Signaling of TNF-α and CD40L through IκBα

In order to see whether TNF-α and/or CD40L affect Ramos cells through the NF-κB pathway, the cells were induced by these cytokines and the levels of IκBα, IκBβ and IκBε were determined by western blotting. *Figure 1*A shows the kinetics of the effects of TNF-α and CD40L on the level of IκBα, in R7 Ramos cells. It can be seen that both cytokines induced a similar pattern of a rapid decrease (within 15 min) in the level of IκBα and partial recovery within the first 2 h. However, these cytokines later induced a second round of reduced level of IKBα. These results corroborate the finding of an oscillating response of the level of IκBα in fibroblasts induced by TNF-α [30]. In five independent experiments CD40L caused a more pronounced initial degradation of IκBα compared to the effect of TNF-α *(figure 1*A*)*. An essentially similar pattern of response to TNF-α and CD40L was obtained for the R1 clone of Ramos (data not shown) and for another BL cell line, BL2 *(figure 1*B*)*. Occasionally, however, CD40L also caused a delayed recovery of IκBα, as shown in *figure 2A*.

Differential signaling of TNF-a and CD40L through IκBβ and IκBε

In order to determine whether TNF-α or CD40L also affects IκBβ or IκBε some of the blots were reprobed with antibodies to IκBβ and IκBε. *Figure 2* shows a comparison of the effects of TNF-α and CD40L on the levels of the three IκB inhibitors. It can be seen that TNF-α caused a relatively slow and gradual decrease in the level of IκBβ over at least 2 h. In contrast, CD40L caused a much more rapid degradation, with almost no recovery, during a 2 h time period *(figure 2B)*.

Figure 2C shows that whereas treatment of Ramos cells with CD40L resulted in a reduction of approximately 60% in the level of IκBε, TNF-α had almost no effect. Cell lysates of untreated and heat shock-treated murine NIH 3T3 and of human HL60 cells served as positive specificity controls for the antibodies used.

It is already known that induction through the CD40 receptor activates the NF-kB pathway in both murine [28] and in human tonsillar B cells and Burkitt's B cell lines [43]. In order to see whether TNF-α induced not only the degradation of IκB proteins, but also led to the translocation of NF-κB protein(s) into the nucleus of Ramos cells, an electrophoretic mobility shift assay was performed. As expected, TNF-α treatment resulted in an increased level of NF-κB complex(es) in the nuclei (beyond the basal constitutive level of nuclear NF-κB in B cells [25]). *Figure 3A* shows that the upper nuclear NF-κB band increased (by 1.5-2 fold) and that the level of the cytosolic upper band correspondingly decreased (approx. 2-fold). All of the bands contained NF-κB complexes since they were successfully competed against by an excess of unlabeled κB oligonucleotide and only partially competed against by a κB oligo mutant (κB*) which lacks NF-κB binding activity. The upper bands in both nuclear and cytosolic extracts contained the NF-κB p65 since it was supershifted with a specific anti-p65 and not by anti-p52 or by a control of rabbit IgG (data not shown). These results indicate that TNF-α activates NF-κB in Ramos cells through the classical pathway mediated by the p65: p50 complex and not by the alternative one mediated by p52: RelB [3]. Western blots of the nuclear extracts of Ramos cells before and after treatment with TNF-α similarly showed an approximately 2-fold increase and 1.5-fold decrease in the level of nuclear and cytosolic NF-κB p65 *(figure 3B)*.

CD40L, but not TNF-α, activates JNK

It is known that TNF-α and CD40L can activate cells not only through the NF-κB pathway but through other signal-

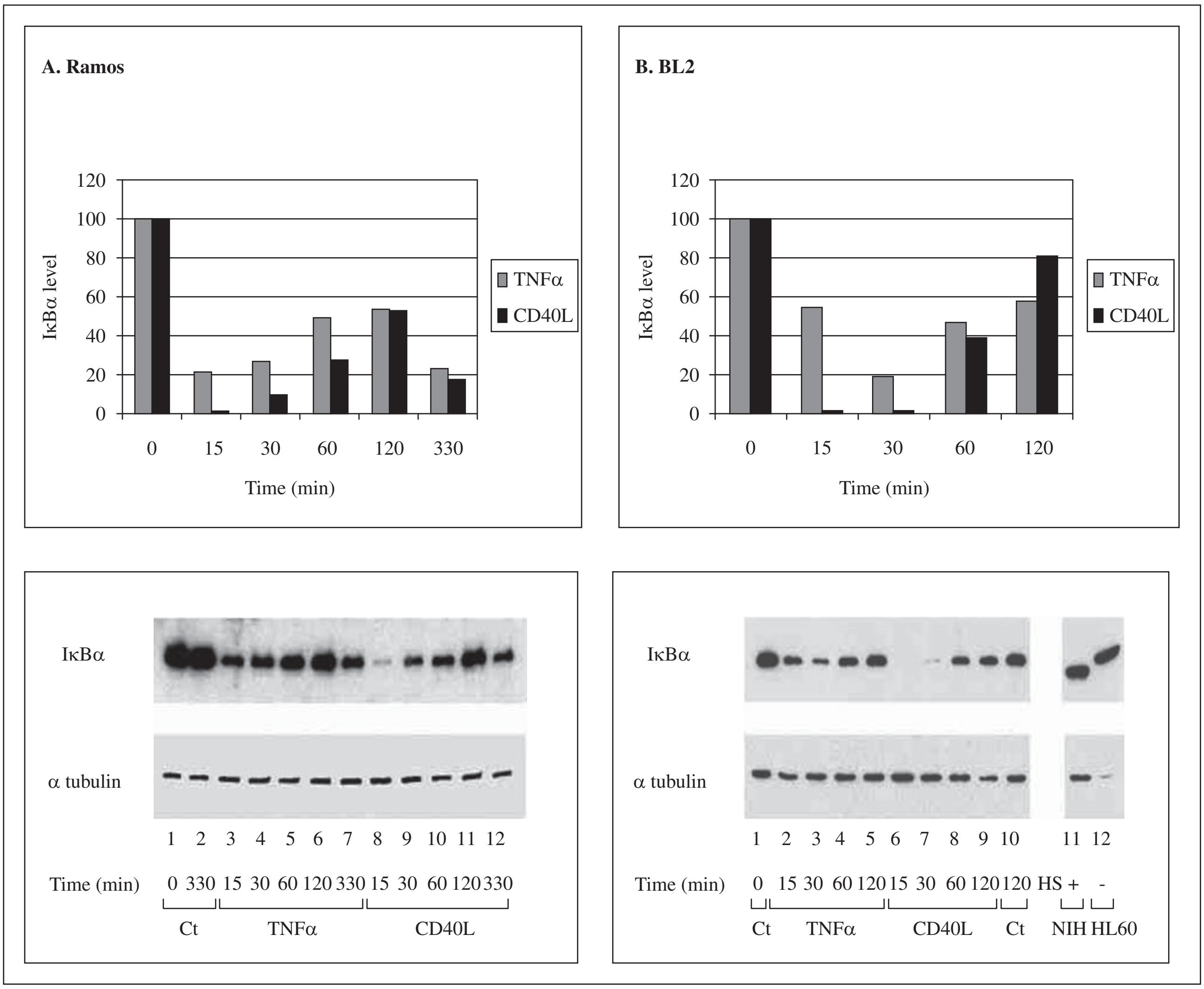

Figure 1
Kinetics of the effects of TNF-α and CD40L on the level of IκBα in R7 Ramos (**A**) and BL2 cells (**B**).
Logarithmically growing cells were induced at 10^6 cells/mL by TNF-α (10 ng/mL) or CD40L (270 ng/mL). The cells were harvested at different time points, washed in IMDM, pelleted, lysed and analysed by western blotting. The membranes were probed with rabbit anti-IκBα and donkey anti-rabbit Ig HRP and developed by ECL. For loading control, the blots were stripped and reprobed with mouse anti α–tubulin and sheep anti-mouse Ig HRP. The OD values of the scanned bands were corrected by the values of the α-tubulin bands in each of the lanes and the relative amounts were normalized to the values of the untreated control cells (Ct). Data in A is representative of four independent kinetic experiments performed for up to the 2 h time point. Murine NIH-3T3 cell lysates from heat shock-treated cells (HS) and from human HL60 cells served as antibody specificity controls (B, lanes 11,12). The murine IκBα band (NIH cells) is smaller in size than the corresponding human HL60 IκBα band (see Santa Cruz cat. 2004, p.104).

ing cascades such as P38, PI3K and JNK, also designated as stress-activated kinase (SAPK) [1, 18]. The family of JNK kinase isoforms is known to activate c-Jun, which participates in the formation of the AP-1 transcription complex responsible for regulation of cell proliferation [44]. In addition, the JNK cascade plays a role in apoptosis of lymphoid cells induced by TNF-α during inflammatory responses [45]. Previous studies have shown that the CD40 receptor could activate the JNK pathway in both Ramos and murine B cells ([18] and references therein). TNF-α is known to induce JNK in various cell types including fibroblasts, HeLa and 293 embryonic kidney cells [46-48]. In murine B cell lines CD40L and TNF-α were found to induce JNK through different mechanisms [49]. We have thus determined whether TNF-α could also induce JNK in Ramos cells. A representative experiment *(figure 4)* shows that after treatment with CD40L and by using monoclonal antibody to the phosphorylated form of JNK, two inducible bands corresponding to phospho-p46 (JNK1) and phospho-p54 (JNK2) were revealed, one below (J1) and one above (J2), with a constitutively expressed middle band (Jx) which could either be a non-specific band, or due to one of the JNK1/2 alternatively spliced isoforms [44] (*figure 4*, lower panel). Cell extracts from untreated and UV-irradiated NIH 3T3 cells served as antibody-negative and -positive controls and showed two bands of similar size to those induced in Ramos cells (*figure 4*, lanes 11, 12). UV irradiation is known to induce two bands in NIH 3T3 cells: p46 and p54 JNK proteins (originating from JNK1 and JNK2 genes, respectively), which are phosphorylated at their Thr183/Tyr185 residues [50]. In Ramos cells, the induced bands appeared at a maximal level within 15 min of treatment with CD40L (11- and 3.5-fold increase in J1 and J2, respectively) and then decayed, but still remained above the baseline level at 2 h (*figure 4* upper right and left panels). In contrast, almost no effect

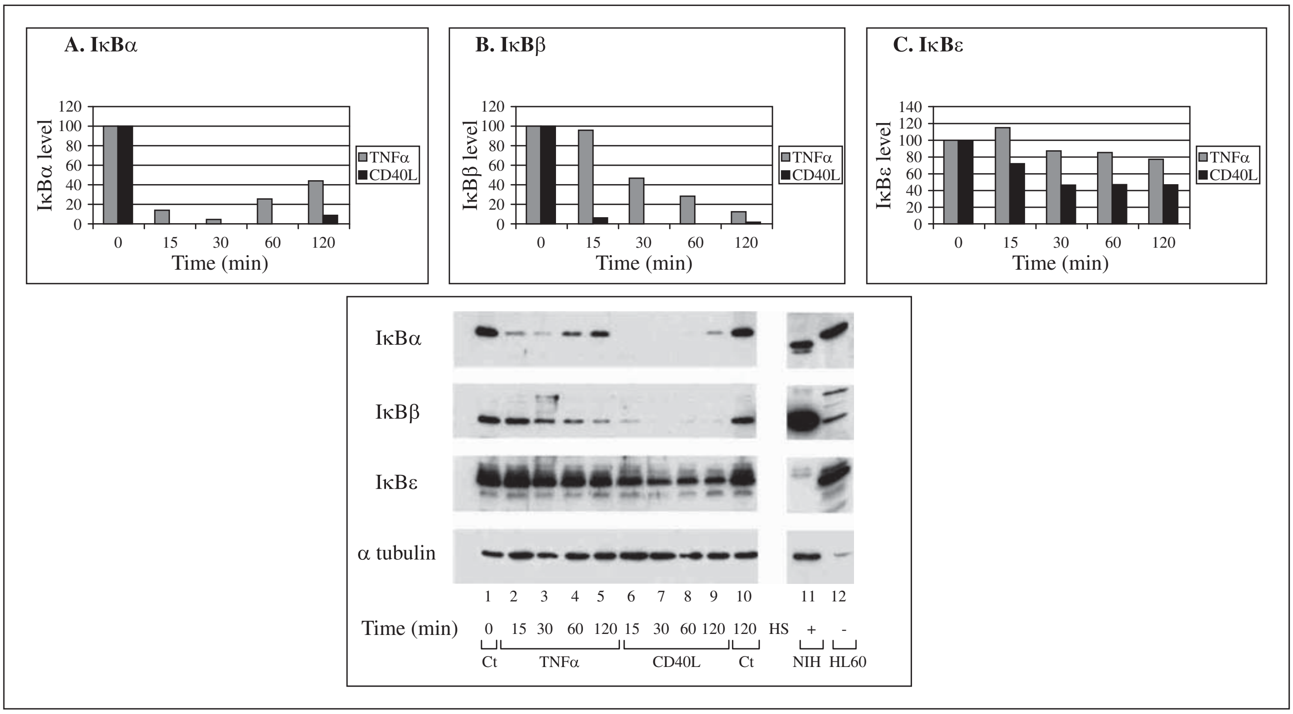

Figure 2

Kinetics of the effects of TNF-α and CD40L on the levels of IκBα (**A**), IκBβ (**B**) and IκBε (**C**) in R7 Ramos cells.

R7 cells (10^6 cells/mL) were induced by TNF-α or CD40L and 10^6 cells were harvested at different time points, lysed and analyzed by western blotting using sequentially rabbit antibodies to IκBα, IκBβ and IκBε. Mouse anti-α tubulin served as loading control. The OD values of the IκB proteins were corrected for loading using the α-tubulin values and normalized for the values of the untreated control cells (Ct). Data in B and C are representative of three independent experiments. NIH-3T3 cell lysates from heat shock-treated (HS) and HL60 cells served as antibody specificity controls (lanes 11,12).

was observed on the level of the middle band (*figure 4*, Jx, upper middle panel), which therefore served as a loading control for the J1 and J2 bands. The latter is also constitutively expressed in THP-1 monocytic leukemia (*figure 4*, lane 13) and in BL2 cells (data not shown). Thus, it appears that in contrast to CD40L, TNF-α does not affect the levels of JNK in Ramos B cells.

Activation of c-Jun by CD40L but not by TNF-α

Since activated JNK can phosphorylate c-Jun [44], we also probed the blots with antibodies to c-Jun phosphorylated at either residue serine 73 (S73) or serine 63 (S63). Multiple inducible bands of phosphorylated c-Jun were detected by antibodies to S73 c-Jun in Ramos cells treated with CD40L, but not with TNF-α (*figure 5* upper panel). These bands probably include c-Jun phosphorylated at S73 and/or S63 and possibly also JunD, which could be phosphorylated (albeit to a lesser extent) at similar sites [44]. UV-induced NIH-3T3 cells served as a positive control and showed two inducible bands of the expected sizes. In contrast, antibody to c-Jun phosphorylated at S63 revealed primarily a constitutively expressed band (or a doublet band) of the expected size in both TNF-α treated and untreated cells. Small increases in the level of S63 c-Jun are noted in the CD40L-induced cells (*figure 5*, middle panel). However, the intensity of the S63 band varied from unchanged to a 2-fold increase in different experiments. A similar S63 c-Jun band was also detected in BL2, another BL cell line (not shown). Altogether, our results show that in Ramos cells, c-Jun is constitutively phosphorylated at S63 and induction by CD40L, but not by TNF-α results in phosphorylation at a new site (S73) of c-Jun. It is possible that phosphorylation at both sites may be required for full activation of c-Jun.

DISCUSSION

In a previous report we found that Ramos cells responded to TNF-α or CD40L by enhanced cell aggregation, enhanced adherence and modulation of cell surface structures such as ruffled membranes and filopodia, without affecting cell viability [40]. In the present study, we have dissected some of the initial events induced by TNF-α and CD40L in the signaling pathways, which may be responsible for the biological effects of these cytokines on Ramos cells. It was found that induction by TNF-αor CD40L resulted in a rapid degradation of IκBα, followed by a period of about 2 hours of partial recovery. Our results are in accord with previous findings which showed that CD40L induces the classical NF-κB pathway in Burkitt's lymphoma cells [28, 34], and show that TNF-α similarly induces the NF-κB pathway in Ramos cells.

The NF-kB pathway has been extensively studied and found to play a critical role in inflammatory and immunological responses [23, 24, 35]. Cellular responses to a variety of ligands and bacterial products, as well as to physical and chemical stimuli, are mediated through this pathway, and multiple ligands, such as IL1, TNF-α Ltx and CD40L of the large TNF-α family, induce the NF-κB pathway through their cognate receptors. These stimuli usually result in activation of the IKK kinase complex, leading to phosphorylation and release of the IκBs from their cytosolic complex(es), which contains the NF-κB

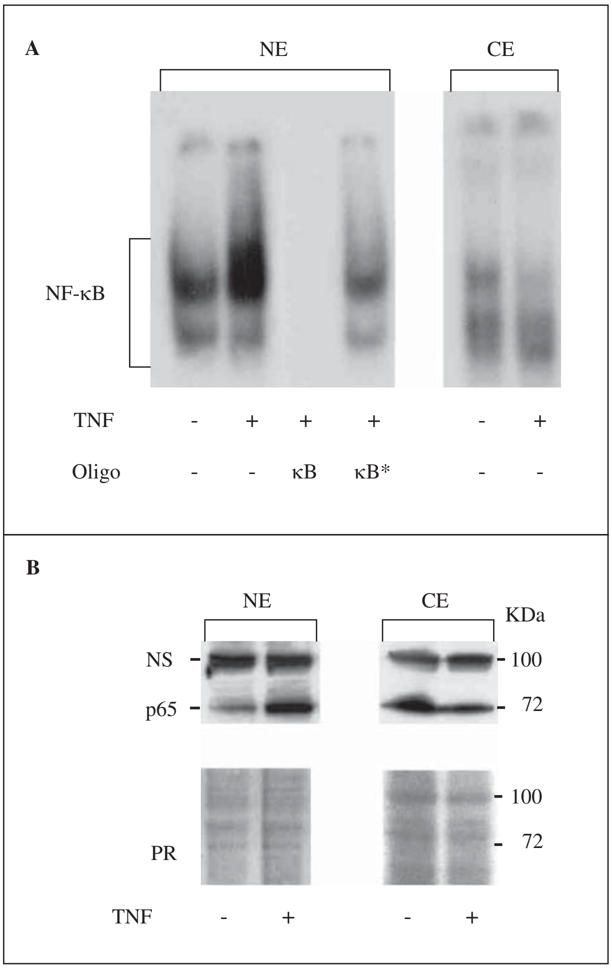

Figure 3

Electrophoretic mobility shift assay (EMSA) and western blots of NF-κB in TNF-α induced Ramos cells. R1 Ramos cells (5x10^6 cells) were induced by 10 ng/mL of TNF-α for 15 min and nuclear and cytosolic extracts were prepared.

A) EMSA assays: Approximately 3x10^4 cpm/ sample of P^{32} - labeled κB oligo probe were mixed with 30 µg of nuclear or cytosolic protein extracts (NE and CE) and the samples were electrophoresed on a 6% polyacrylamide gel. The gel was dried and autoradiographed. The labeled free probe is not shown. A hundred-fold molar excess of cold κB oligo probe, which competes successfully with the labeled probe, and a similar excess of mutated oligo (κB*), which does not bind to NF-κB, were used as specificity controls. **B**) Western blotting: 20 µg of cytosolic (CE) or nuclear (NE) extract proteins were loaded per lane and electrophoresed in 9% SDS-acrylamide gels. The gels were blotted to NC membranes, stained with 0.1% Ponceau red (PR) in 5% acetic acid and photographed (lower panels). Blocking was done with 2% BSA in PBS containing 0.1% Tween 20. The filters were probed with rabbit antibodies to p65, washed and reprobed with goat anti-rabbit IgG HRP(H+L), and developed by ECL (upper panels).

transcription factors. Depending on cell type and stimulus, different combinatorial hetero/homo dimers of the five known NF-κB/Rel transcription factors may then translocate into the nucleus and activate or suppress, respectively, the transcription of overlapping and distinct groups of target genes which contain the κB motif in their regulatory region. Thus, in resting B cells for example, there is a preferential release and nuclear translocation of the p50: RelA dimer in LPS type 1- induced NF-κB responses (i.e., the classical NF-κB pathway), whereas the p52: RelB dimer is affected in BAFF type 2 (alternative) NF-κB response [28]. Moreover, a basal level of the NF-κB signaling pathway is constitutively activated in B lymphocytes in which NF-κB complexes already reside in the nucleus with no known inducing signal [25, 51, 52].

The NF-κB system is even more complex, since in addition to IkBα, other IkBs are known to regulate and affect the pathway (for example, IkBβ and IkBε) [35, 53]. We have therefore also examined in Ramos the kinetics of the effect of TNF-α and CD40L on the levels of IκBβ and IκBε. Our results showed that the two cytokines each have differential effects on the degradation and recovery patterns of IκBβ and IκBε. Differential responses to LPS and TNF-α were previously reported for IκBα, IκBβ and IκBε in non-B cells such as monocytes [30, 54], and distinct responses of IκB proteins to TNF-α were also demonstrated in fibroblasts and T cells [30]. For example, the previously described responses of the IκBβ and IκBε to TNF-α in monocytes [54] were quite different from the corresponding responses to TNF-α in Ramos B cells as found in the present work. On the other hand, we corroborated in B cells previous findings made in Jurkat T cells, showing that TNF-α induces a rapid degradation of IκBα, but a slow and gradual degradation of IκBβ [55]. These observations, combined with the finding that IκBα recovers rapidly and that IκBβ has a much slower recovery period, led to the suggestion that while IκBα controls rapid NF-κB cellular responses, IκBβ is responsible for long-lasting responses. However, our results show that this is not always the case since in the same Ramos cells, both IκBα and IκBβ were rapidly degraded following induction by a different ligand, CD40L. Our findings thus demonstrate that ligand specificity may play a critical role in determining the specificity of NF-κB responses mediated through the IκB proteins.

In contrast to our findings in Ramos cells that IκBε is minimally affected by TNF-α this cytokine induced a rapid degradation of IκBε accompanied by a very slow recovery period in murine fibroblasts and in human monocytes [30, 54]. Interestingly, in murine lung tissue TNF-α was even found to markedly increase the level of IkBε [56]. Altogether, these findings demonstrate that cell-type specificity can strongly affect the outcome of NF-κB responses mediated by different IκB isoforms.

Our combined results thus show that even in the same cell, differential regulatory responses to TNF-α *versus* CD40L exist, and that the IκB family members tested may respond differentially to these cytokines. Overall, the patterns of degradation and recovery of the IκB proteins seem to be ligand- and cell-type specific. Mechanistically, the simplest explanation of our observations is that even in a single type of cell, multiple functionally distinct NF-κB complexes composed of different IκB molecules exist, thus allowing differential responses to a multitude of external stimuli. This model is similar to that proposed by Cheshire and Baldwin [57] to explain the synergistic activation of NF-κB by TNF-α and IFN-γ *via* degradation of IκBα and IκBβ.

Because of their structural similarities and the occurrence of two similar serine phosphorylation sites in their N-terminal region (*i.e.,* S32 and S36), the question of why IκBα is rapidly degraded and IκBβ degrades slowly following induction by TNF-α is not fully resolved [53]. It is possible that the efficiency of phosphorylation of the two proteins by the same upstream IKKkinase complex differs, and/or that there is an additional (not yet known) IκBβ specific kinase which is responsible for the observed slow

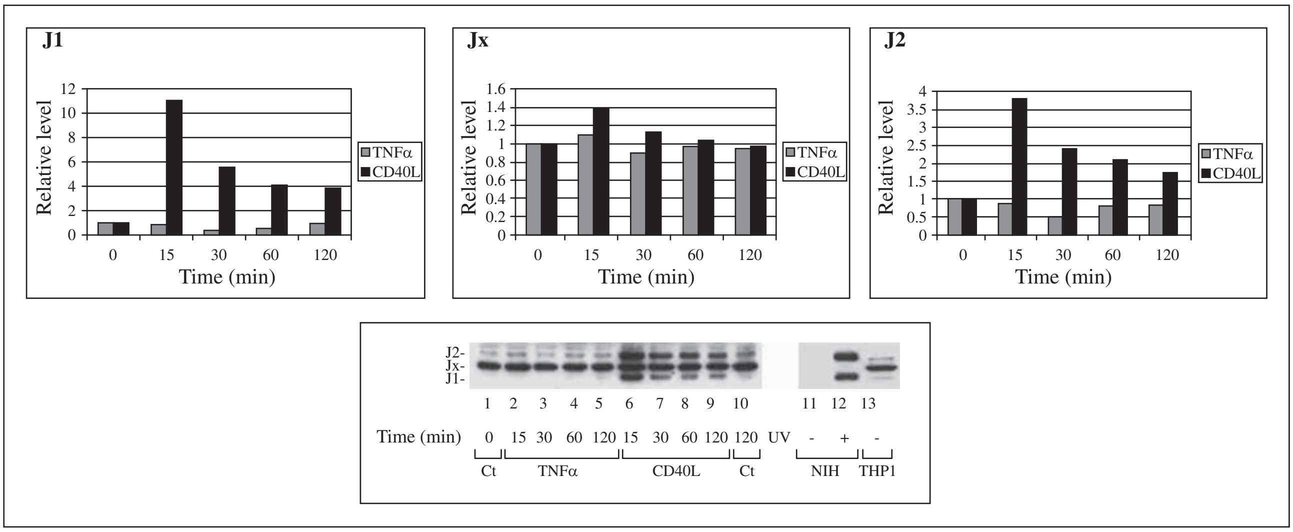

Figure 4
Kinetics of the effects of TNF-α and CD40L at the level of phosphorylated SAPK/JNK in R7 Ramos cells.
The cells (10^6 cells/mL) were induced by TNF-α or CD40L and aliquots of 10^6 cells were collected at different time intervals, washed, lysed and analyzed by western blotting. The filters were probed with mouse anti-phosphorylated JNK. Two inducible bands, J1 and J2, are apparent following CD40L, but not TNF-α treatment (lower panel). The relative level of the Jx band was determined as the ratio of the Jx band intensity at each of the time points to the mean of the Jx band intensity in the controls (Ct) at times 0 and 120 min. Since the middle Jx band did not change much and seems to be constitutively expressed (upper middle panel), it served as a loading control. The relative intensities of the J1 and J2 bands (upper right and left panels) were similarly determined for each of the time points as the ratio of the J1 or J2 band intensities to the mean of their corresponding values of the untreated controls (Ct at times 0 and 120 min). These values were then normalized by the relative values of the corresponding Jx middle bands. Lysates derived from untreated (-) and UV-treated (+) NIH-3T3 and HL60 cells were used as antibody specificity controls (lanes 11-13). Data are representative of at least three independent experiments.

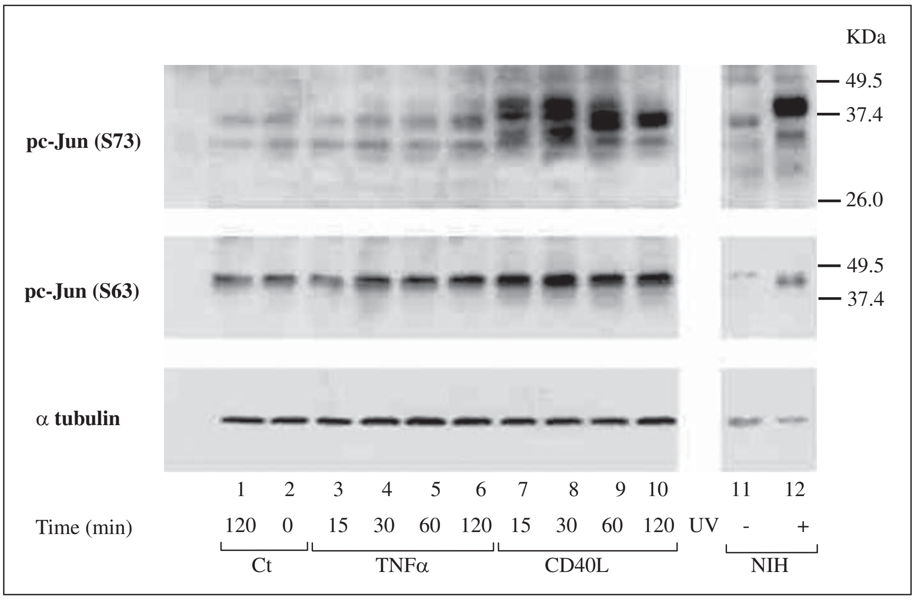

Figure 5
Kinetics of the effects of TNF-α and CD40L on the level of phosphorylated c-Jun in R7 Ramos cells. Cells (10^6 cells/mL) were induced by TNF-α or CD40L for up to 2 h and samples of 10^6 cells were lysed at different time points and analyzed by western blotting. The blotted filter was sequentially probed with mouse anti-phosphorylated c-Jun (S63) (middle panel), developed, stripped and reprobed with rabbit anti-phosphorylated c-Jun (S73) (upper panel) and finally with mouse anti α-tubulin, which served as a loading control (lower panel). Lysates of NIH-3T3 cells prepared before (-) and following UV treatment (+) were used as antibody specificity controls (lanes 11, 12). Data are representative of at least three independent experiments.

degradation rate of IκBβ. Alternatively, the slower degradation rate may be due to other steps such as ubiquitination or proteasome degradation in the canonical IκB degradation pathway. In addition, IκBβ may be modified by phosphorylation at a site(s) in its C-terminal region [35], and this may affect its rate of degradation either by the classical proteasome pathway and/or by one of the alternative cellular degradation pathways [58]. It was recently found that IκBβ, but not IκBα could form a novel type of complex with NF-κB and κB-ras proteins and that this complex stabilizes and prevents induced degradation of IkBβ [59]. Our findings that the rate of IκBβ degradation in Ramos can be markedly altered, depending on the inducing ligand (*i.e.,* TNF-α or CD40L), are in accord with this observation and support the notion that there is more than one route of degradation of the IκBβ molecule.

As for IκBα there also seem to be several mechanisms for its release/degradation from its complex with NF-κB. In addition to the classical pathway of phosphorylation at Ser32 and Ser36, followed by ubiquitination and proteasome degradation, there is evidence in non-B cells for Ca^{++}-activated, calpain-dependent degradation without prior phosphorylation of the Ser32/Ser36 sites of IκBα [58, 60], and of phosphorylation of Tyr42 causing IκBα dissociation (and p65 nuclear translocation) without prior IκBα degradation, in response to hypoxia or to pervandate and silica stress signals [61, 62]. Moreover, in mature murine B cells the constitutive turnover of IκBα, but not of IκBβ, was found to rely on an alternative, non-canonical, degradation pathway [52].

CD40L is known to activate multiple cellular signaling pathways in B cells through its cognate CD40 receptor [2]. The NF-κB and the JNK pathways, which are activated through TNFR-associated factors (TRAF) 2, 5 and 6, but not by TRAF 1, 3 and 4 proteins, have been extensively studied [19]. TNF-α also induces multiple pathways in B cells, some of which are similar to those induced by CD40L [1].

A striking difference was found in the effects of TNF-α versus CD40L. Whereas treatment with CD40L caused activation of the amino-terminal JNK kinase and phosphorylation of its target proteins c-Jun and Jun D, TNF-α failed to have such an effect. In contrast, in murine B cells both CD40L and TNF-α were found to activate the NF-κB and JNK cascades [49]. This difference in the response to TNF-α may be due to the expression of TNFR2, but not of TNFR1, and activation of the NF-κB cascade through the alternative pathway in the studied murine B cells [49]. By contrast, Ramos cells mainly express TNFR1 [40], and as found in the present work, activate NF-κB through the classical pathway by degradation of IκBα/IκBβ. It remains to be seen whether these different responses to TNF-α reflect the differentiation states of the murine versus the human B cell lines involved.

Since the JNK pathway is induced by CD40L, but not by TNF-α, and both were found to initiate signaling of the NF-κB pathway through degradation of IκBα and/or IκBβ, it seems reasonable to assume that activation of the NF-κB cascade is responsible for the observed phenotypic modulation of Ramos cells induced by these cytokines [40]. However, in preliminary experiments we have tried to inhibit the NF-κB pathway by two known synthetic inhibitors of the pathway: BAY11-7085 [63] and caffeic acid phenethyl esther (CAPE) [64]. It was unexpectedly found that both BAY and CAPE caused cell death at IC50s of about 1 μM and 4 μM, respectively, and equal or lower concentrations of these drugs did not significantly inhibit the induction by CD40L or TNF-α of the pattern of cell aggregation or the induced modification of cell morphology (Nir B and Laskov R, in preparation). These findings imply that other signaling cascades may be involved in the phenotypic modulations observed in Ramos cells treated with TNF-α and CD40L.

Even though both Ramos and BL2 are EBV-negative Burkitt's lymphoma cell lines, the two cell lines behaved differently. Whereas Ramos cells aggregated spontaneously and responded to either TNF-α or CD40L by enhanced aggregation and modulation of cell surface morphology, BL2 grew in single-cell suspension and did not respond to these cytokines either in modulating its surface morphology or in its growth behavior [40]. However, at the biochemical level, both reacted and were induced by TNF-α and CD40L, even though there were quantitative differences in their response to these cytokines: compared to Ramos, IκBα was less degradable in BL2 following treatment with either CD40L or TNF-α and JNK and c-Jun were not phosphorylated in response to CD40L in BL2 cells. More work is necessary in order to find out if these differences in biological and biochemical responses are related to the findings that SHM of the rearranged IgV genes is constitutive in Ramos [17] and can be induced in BL2 cells [16].

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