

# Lymphoid B cells induce NF- $\kappa$ B activation in high endothelial cells from human tonsils

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## Abstract

**Immune surveillance depends on still poorly understood lymphocyte–endothelium interactions required for lymphocyte transendothelial migration into secondary lymphoid organs. The nuclear factor  $\kappa$ B (NF- $\kappa$ B) regulatory system and its inhibitory I $\kappa$ B proteins control the inducible expression of adhesion molecules, cytokines and chemokines involved in endothelial activation and lymphocyte transmigration. Here we present results showing the activation of this system in response to the interaction of high endothelial cells from human tonsils (HUTEC) with human B and T lymphoid cell lines and primary tonsillar lymphocytes. Western blot and electrophoretic mobility shift assays show that adhesion of different lymphoid cells induce varying levels of NF- $\kappa$ B activation in HUTEC, with Daudi cells, tonsil-derived B cell line 10 (TBCL-10) and primary tonsillar B lymphocytes causing the strongest activation. The main NF- $\kappa$ B protein complexes translocated to the nucleus were p65/p50 and p50/p50. Results from reverse transcription–PCR and flow cytometry analysis of HUTEC indicate that the interaction with Daudi cells induce an increased expression of IL-6 and IL-8 mRNA and cell-surface expression of intercellular adhesion molecule-1, all of which were prevented by sodium salicylate, an inhibitor of NF- $\kappa$ B activation. Transwell experiments show that NF- $\kappa$ B activation and the response of HUTEC to the interaction of Daudi cells does not depend on direct cell–cell contact but rather on the production of soluble factors that require the presence of both cell types. These results suggest that lymphocytes and high endothelium establish a cross talk leading to NF- $\kappa$ B-mediated expression of cytokines and adhesion molecules, inducing endothelial cell activation.**

## Introduction

Endothelial cells play an active role in vascular physiology driving and modulating a number of biological processes including leukocyte traffic (1). It is now well known that during inflammation, endothelial cells and leukocytes establish a two-way process of information transfer leading to their mutual activation and increased leukocyte extravasation (2–4). Lymphocyte recruitment and migration to the sites of inflammation involve triggering of adhesive properties, mediated by specific signaling events, occurring in both lymphocytes and endothelial cells [for review, see Springer (5, 6) and

Butcher and Picker (7)]. As part of the immune surveillance process, lymphocytes continuously extravasate through specialized post-capillary high endothelial venules (HEVs) of secondary lymphoid organs, such as lymph nodes, Peyer's patches and tonsils. HEVs display special structural and functional features indicative of a permanently activated state which allows them to support high rates of lymphocyte extravasation not found in endothelial cells lining other vessels [reviewed in Girard and Springer (8)]. In contrast to the many reports on inflamed endothelium, the mechanisms involved

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in lymphocyte/HEV communication implicated during normal lymphocyte transmigration have remained relatively unexplored. A particular aspect addressed in the present work deals with the participation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) transcription factors in this process.

Triggering of endothelial cell activation can be induced in a variety of processes, including atherogenesis, angiogenesis and inflammation, and requires the expression of specific genes that lead to a number of endothelial phenotypic changes (1). A main regulator of gene expression during endothelial cell activation is the ubiquitous NF- $\kappa$ B family of transcription factors (9). The NF- $\kappa$ B regulatory system is composed of the structurally related proteins p65 (Rel A), c-Rel, Rel B, p50/p105 and p52/p100 [for review, see Baldwin (10) and May and Ghosh (11)]. p65, c-Rel and Rel B contain potent transactivation domains. In unstimulated cells, homo- or heterodimers of NF- $\kappa$ B are retained in the cytoplasm in an inactive form through interaction with the family of inhibitory proteins I $\kappa$ Bs, which impede their nuclear translocation. In response to a variety of stimuli, I $\kappa$ B proteins undergo phosphorylation and rapid degradation via proteasome, thus releasing NF- $\kappa$ B transcription factors that can now translocate to the nucleus where they activate genes bearing  $\kappa$ B DNA-binding sites in their promoter regions. NF- $\kappa$ B regulates the endothelial expression of adhesion molecules, such as E-selectin (12), intercellular adhesion molecule-1 (ICAM-1) (13) and vascular cell adhesion molecule-1 (14, 15), as well as cytokines such as IL-6 (16) and chemokines such as IL-8 (17) and monocyte chemoattractant protein-1 (MCP-1) (18). All these molecules participate actively in the transendothelial migration of lymphocytes in response to inflammatory stimuli.

Using a model system of high endothelial cells from human tonsils (HUTEc) (19, 20) we have previously reported that adhesion with B lymphocyte resulted in cytokine and chemokine production through a cascade of protein phosphorylation that included FAK125, paxillin and ERK2 (20). In the present study, we asked whether the interaction between high endothelium and lymphoid cells also resulted in the activation of the NF- $\kappa$ B regulatory system in these cells. Our results show that HUTEcs respond to the interaction with lymphoid B cells (but not with T cells) by inducing NF- $\kappa$ B activation, which in turn controls the increased expression of IL-6 and IL-8 mRNAs and ICAM-1 surface protein. HUTEc activation occurred through the production of soluble factors that required the presence of both endothelial cells and lymphocytes, suggesting a mechanism of cross talk and reciprocal cell signaling. Thus, lymphoid B cells interact with high endothelial cells activating the NF- $\kappa$ B system leading to the activation of the endothelium, an event that may be required for the differentiation or permanent activation of high endothelium.

## **Methods**

### *Cells*

HUTEc were prepared from tonsils obtained from children undergoing programmed tonsillectomy, as previously described (19). Allogeneic tonsillar lymphocytes (ToL) were released from tonsil by digestion with 400  $\mu$ g ml<sup>-1</sup> collagenase XI (Sigma Chemical Co., St Louis, MO, USA) and 100  $\mu$ g ml<sup>-1</sup> DNase I (Sigma) for 1 h at room temperature. The cell

suspension was washed three times in RPMI 1640 medium (GIBCO-BRL, Rockville, MD, USA), supplemented with 10% FCS (GIBCO-BRL) and lymphocytes were isolated by Ficoll-Hystopaque gradient centrifugation. Tonsillar B cells were prepared after depleting ToL of T cells with the OKT-3 mAb [American Type Culture Collection (ATCC), Rockville, MD, USA] and complement, while a fraction enriched in T cells was obtained after treatment of ToL with the L112 (21) mAb and complement. Flow cytometry analysis showed that purified tonsillar B lymphocytes contained <8% of T cells, while the T cell fraction exhibited a B cell contamination <2.6%.

Human B cell lines, Daudi and Ramos, and T cell lines, Jurkat and Molt-4, were obtained from ATCC. Tonsil derived B-cell-line-10 (TBCL-10) is a cell line obtained in our laboratory from human tonsil B cells transformed with EBV (20). All these cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM of L-glutamine, 100 U ml<sup>-1</sup> of penicillin and 100  $\mu$ g ml<sup>-1</sup> of streptomycin (all reagents are from GIBCO-BRL).

### *Reagents and antibodies*

Antibodies directed against Rel proteins (anti-p65, anti-p50 and anti-c-Rel), anti-transcription factor IIB (TFIIB), anti-c-Raf-1 and anti- $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAb anti-ICAM-1-FITC and isotype control Ig were from Immunotech (Marseille, France). Peroxidase-conjugated anti-rabbit, anti-mouse and anti-goat Ig were from Promega (Madison, WI, USA).

Recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was from R&D Systems (Minneapolis, MN, USA). Sodium salicylate (NaSal) and *N*-acetyl-L-cysteine (NAC) were from Sigma. Indomethacin was purchased from Cayman Chemical (Ann Arbor, MI, USA).

### *Co-cultures and cell extracts*

HUTEcs from passages 3–5 were grown to confluence in 100-mm plates ( $2\text{--}3 \times 10^6$  cells). After washing with RPMI 1640, the culture medium was replaced by fresh medium and the HUTEc incubated for an additional 1 h in order to minimize NF- $\kappa$ B basal activation caused by cell culture manipulation. Co-cultures were started by adding 100  $\mu$ l of the following cell suspensions: lymphoid cell lines ( $10 \times 10^6$  cells), ToL ( $5 \times 10^6$  cells), purified T cells ( $10 \times 10^6$  cells) and purified B cells ( $5 \times 10^6$  cells) for different periods of time as indicated. Co-culture experiments in the presence of NaSal, NAC or indomethacin were performed by pre-incubating HUTEc with these drugs for 1 h and then adding Daudi cells to the cultures for an additional 3 h. For experiments in Transwell cell culture chambers (24-mm-diameter wells, 0.4- $\mu$ m pore size, Costar, Cambridge, MA, USA), Daudi cells ( $3 \times 10^6$ ) were added for different periods of time to confluent monolayers of HUTEc ( $0.8\text{--}1 \times 10^6$ ) grown either in the lower well or in the upper chamber as indicated. In other experiments, cells were co-cultured in separate compartments, with Daudi cells in the upper chamber and HUTEc in the lower well. After the incubation period, the culture medium was recovered and centrifuged at  $250 \times g$  and the supernatant was frozen at  $-80^\circ\text{C}$  for further analysis. HUTEc were washed with ice-cold PBS and bound lymphocytes were removed by pipetting until no lymphocytes were detected by light microscopy (20). In control experiments, untreated HUTEcs were subjected to

the same manipulations to exclude artifacts that may have been produced by the washing process. Cytosolic extracts were obtained by scraping HUTEc in 400  $\mu$ l of buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM ethylene-diamine-tetraacetic-acid (EDTA), 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride (PMSF), 1  $\mu$ g ml<sup>-1</sup> pepstatin A, 1  $\mu$ g ml<sup>-1</sup> leupeptin, 1  $\mu$ g ml<sup>-1</sup> aprotinin] and incubated on ice for 15 min. After adding Nonidet P-40 at 0.5% final concentration, the cell suspension was vortexed for 30 s and centrifuged at 12 000  $\times$  *g* for 30 s at 4°C. The supernatant containing the cytosolic extract was aliquoted and stored at -80°C for subsequent analysis. Nuclear extracts were obtained by re-suspending the pellet in 50  $\mu$ l of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1  $\mu$ g ml<sup>-1</sup> pepstatin A, 1  $\mu$ g ml<sup>-1</sup> leupeptin, 1  $\mu$ g ml<sup>-1</sup> aprotinin) and placed on a roller shaker for 15 min at 4°C. The homogenate was centrifuged at 12 000  $\times$  *g* for 5 min at 4°C and the supernatant was recovered, aliquoted and stored at -80°C for subsequent analysis.

#### *Western blot analysis*

For immunoblots, 12  $\mu$ g of cell extract protein (determined using the Bradford assay; Bio-Rad, Hercules, CA, USA) was separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose sheets in 25 mM Tris, 192 mM glycine and 5% methanol at 450 mA for 1 h at 4°C. The blots were blocked in PBS containing 10% non-fat powdered milk for 1 h at room temperature and then incubated with the primary antibody (1:1000) for 1 h. After washing the membranes three times in PBS with 0.2% Tween 20, the blots were incubated with the appropriate HRP-linked secondary antibody (1:2500) (Promega) for 1 h at room temperature. Immunoreactive proteins were detected by the enhanced chemiluminescent protocol (Amersham, Arlington Heights, IL, USA). Afterward, the blots were stripped in 0.1 M glycine, pH 2–3, for 15 min at room temperature followed by incubation in 1 M NaCl/PBS for 2 min. After washing twice in PBS containing 0.2% Tween 20, the blots were blocked again as indicated above and immunostained with a new antibody.

#### *Electrophoretic mobility shift assay*

A probe (5'-CAACGGCAGGGGAATCCCCTCTCCTT-3' plus 5'-AAGGAGAGGG-3') containing a consensus  $\kappa$ B site was end-labeled with  $\alpha$ -[<sup>32</sup>P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase I as described in Burgos *et al.* (22). Binding reactions were performed with 2  $\mu$ g of nuclear extract protein in 20  $\mu$ l binding buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol), 2  $\mu$ g poly dI-dC (Pharmacia) and 100 000 c.p.m. of <sup>32</sup>P-labeled probe. Reaction mixtures were incubated at room temperature for 30 min and analyzed by electrophoresis on a 6% non-denaturing polyacrylamide gel in 0.05 M Tris-borate and 1 M EDTA at 120 V for 2.5 h. After electrophoresis, gels were dried and DNA-protein complexes localized by autoradiography. Competition studies were performed by adding unlabeled probe to the binding reaction. For supershift analysis, nuclear extracts were incubated with 2  $\mu$ l of specific anti-sera or non-immune rabbit serum for 1 h on ice before the addition of binding buffer containing the labeled probe. Samples were subjected to electrophoresis as described above.

#### *Reverse transcription-PCR*

In the co-culture experiments, RNA was extracted from HUTEc and Daudi cells by using Trizol reagent as suggested by the manufacturer (GIBCO-BRL). cDNA synthesis was carried out for 1 h at 42°C by incubating 5  $\mu$ g of RNA, 500 ng of oligo(dT) (Biosource, Camarillo, CA, USA), 200 U mouse Moloney leukemia virus (MMLV)-reverse transcriptase (Promega), 24 U RNasin (Promega) and 0.5 mM deoxynucleoside triphosphate (dNTP) (GIBCO-BRL) dissolved in MMLV-RT buffer to 25  $\mu$ l final volume. PCRs were performed in a Minicycler (MJ Research) on a reaction mix containing 2  $\mu$ l of the resulting cDNAs, 200 ng of the sense and antisense primers, 200  $\mu$ M dNTP, 1.5 mM MgCl<sub>2</sub> (GIBCO-BRL) and 5 U *Taq* DNA polymerase (GIBCO-BRL) in AmpliTaq buffer to 25  $\mu$ l final volume. Primers were designed to span intron-exon junctions to differentiate between cDNA and genomic DNA. For IL-6 and  $\beta$ -actin mRNA amplification we used the following conditions: one cycle of 5 min at 95°C and 20 cycles of 30 s at 94°C, 45 s at 58°C and 90 s at 72°C. For IL-8 we used an initial cycle of 5 min at 95°C, followed by 25 cycles of 1 min at 94°C, 90 s at 60°C and 90 s at 72°C. All programs were finished by a final extension of 10 min at 72°C. Ten microliters of the mixture was electrophoresed in a 1.4% agarose gel and stained with ethidium bromide to visualize the amplification product. Specific primers for IL-6 and  $\beta$ -actin were as previously described (23, 24). Primers for IL-8 were 5'-ATTTCTGCAGCTCTGTGTGAA-3' sense and 5'-CCTACA-ACAGACCCACACAAT-3' antisense.

#### *Flow cytometry analysis*

Cell-surface expression of ICAM-1 on HUTEc was determined by indirect immunofluorescence assays, as previously detailed (19). Fluorescence analysis was performed with a FACScan and Cellquest software (Becton Dickinson, San José, CA, USA).

#### *TNF- $\alpha$ - and lymphotoxin-sensitive bioassay*

TNF- $\alpha$  and lymphotoxin were quantified by evaluating the cytotoxicity of culture supernatants over actinomycin D-treated murine fibroblast L929. For this, L929 cells were plated in 96-well culture plates at 2  $\times$  10<sup>4</sup> cells per well and incubated overnight at 37°C and 10% CO<sub>2</sub>. The medium was then removed and 50  $\mu$ l of test or control supernatants was added to the cells, followed by the addition of 50  $\mu$ l of a stock actinomycin D solution (8  $\mu$ g ml<sup>-1</sup>). The cells were incubated overnight at 37°C and 10% CO<sub>2</sub>, washed with PBS, stained with 50  $\mu$ l of 0.05% crystal violet in 20% ethanol for 10 min at room temperature, washed with water and allowed to dry. To evaluate cell staining, 100  $\mu$ l of methanol was added to each well and the optical density read on a microtiter plate reader at 595 nm (25). Recombinant human TNF- $\alpha$  was used as positive control. Each assay was done in triplicate.

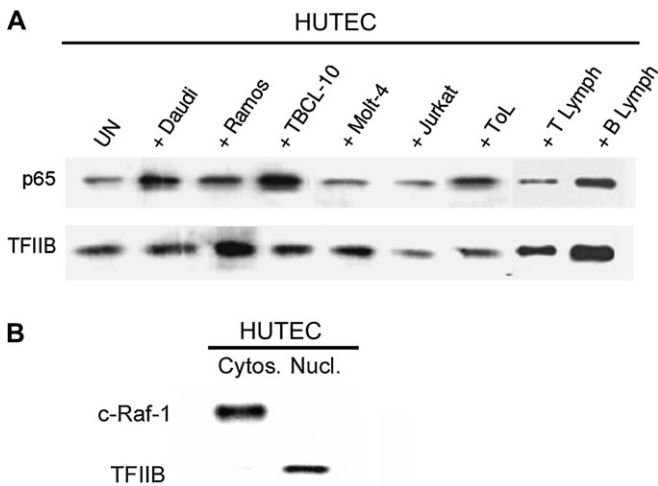
## **Results**

### *Lymphocytes induce NF- $\kappa$ B activation in HUTEc*

It has been widely documented that NF- $\kappa$ B is implicated in endothelial cell activation (9, 26–28). To determine whether

### Lymphocyte-induced high endothelial cell activation

NF- $\kappa$ B could also be activated by the interaction of high endothelial cells with lymphocytes, we analyzed by western blot the levels of p65 protein in nuclear extracts prepared from HUVEC incubated with different T and B lymphoid cell lines as well as with isolated total allogeneic ToL, and purified allogeneic tonsillar B and T lymphocytes. Figure 1(A) shows that ToL, primary tonsillar B lymphocytes as well as the B cell lines induced a strong NF- $\kappa$ B activation, with Daudi and TBCL-10 cells showing the greater effect. On the other hand, purified tonsil-derived T lymphocytes and the T cell lines Molt-4 and Jurkat had no effect, presenting p65 levels similar to the untreated control (Fig. 1A). The purity of the nuclear cell extracts was established using antibodies to specific cytoplasmic (c-Raf-1) and nuclear (TFIIB) proteins (Fig. 1B). To rule out that the changes in p65 were due to B lymphoid cells remaining in the cultures, we determined the proportion of these cells in the cultures after the washing steps as well as the levels of p65 presumably contributed by the remaining B cells. Since flow cytometry analysis using the CD20 B cell-specific marker indicated that residual contamination by Daudi cells represented <10% of the initial number of Daudi cells added to the cultures (data not shown), we performed western blot experiments of a control sample supplemented with an amount of protein equivalent to 10% of Daudi cells. These experiments showed that p65 could not be detected under these conditions (data not shown). Lymphocyte-induced activation of NF- $\kappa$ B in HUVEC was corroborated by electrophoretic mobility shift assay (EMSA) (Fig. 2), in which Daudi and TBCL-10 cells showed the strongest  $\kappa$ B probe binding activity. Also, primary ToL induced a significant NF- $\kappa$ B activation.

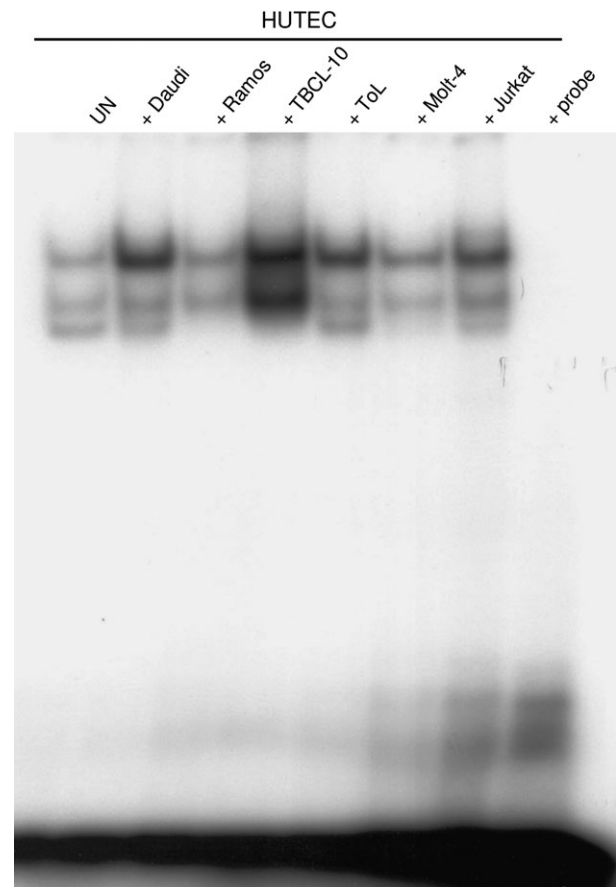


**Fig. 1.** B lymphocytes induce NF- $\kappa$ B activation in HUVEC. (A) Nuclear extracts from untreated (UN) HUVEC, co-cultured with B cell lines ( $10 \times 10^6$  cells; Daudi, Ramos, TBCL-10), T cell lines ( $10 \times 10^6$  cells; Molt-4, Jurkat) and primary allogeneic ToL ( $5 \times 10^6$  cells), T lymphocytes ( $10 \times 10^6$ ; T Lymph) and B lymphocytes ( $5 \times 10^6$ ; B Lymph) for 3 h were immunoblotted with anti-sera to the p65 subunit of NF- $\kappa$ B. The blots were stripped and immunostained for TFIIB, which was used as a control of protein loading. The results are representative from three independent experiments. (B) Cytosolic (Cytos) and nuclear (Nucl) extracts prepared from HUVEC were immunoblotted with anti-sera to c-Raf-1 and TFIIB.

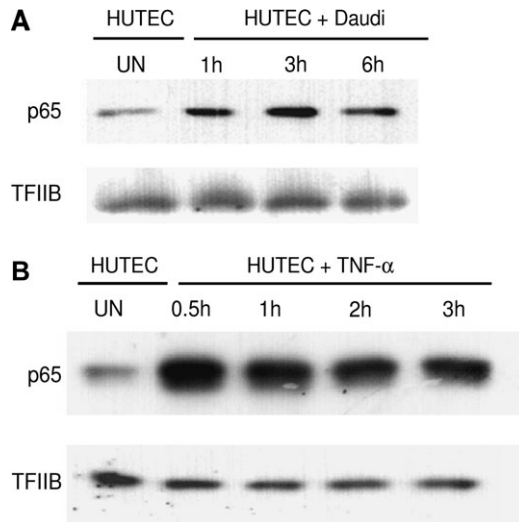
### Time course of NF- $\kappa$ B activation in HUVEC co-cultured with Daudi

Based on the results obtained with the purified primary tonsillar B lymphocytes and the different B cell lines, we chose Daudi cells, a cell line described not to secrete lymphotoxin (29) and a potent NF- $\kappa$ B activator, to further characterize the induction of NF- $\kappa$ B observed in HUVEC after incubation with B lymphoid cells. Although TBCL-10 cells induced a stronger NF- $\kappa$ B activation than Daudi cells, they have the disadvantage of secreting TNF- $\alpha$  and/or lymphotoxin (see below), two well-known NF- $\kappa$ B activators.

Upon incubation with Daudi cells, HUVEC showed a time-dependent increase in NF- $\kappa$ B activation with maximal activation reached after 3 h (Fig. 3A). For comparative purposes, we determined the time course of NF- $\kappa$ B activation in HUVEC treated with TNF- $\alpha$ . Figure 3(B) shows that 0.5 h of TNF- $\alpha$  treatment induced a strong increase in nuclear p65, which remained unchanged for 1 h, subsequently decreasing at 2 and 3 h. Therefore, in comparison to the NF- $\kappa$ B activation induced by TNF- $\alpha$ , the interaction of Daudi cells with HUVEC induced a slower NF- $\kappa$ B activation.



**Fig. 2.** NF- $\kappa$ B binding activity in nuclear extracts of HUVEC co-cultured with lymphoid cells. Nuclear extracts from untreated (UN) HUVEC or co-cultured with B cell lines (Daudi, Ramos, TBCL-10), with allogeneic ToL or with T cell lines (Molt-4, Jurkat) for 3 h were incubated with a  $^{32}$ P-labeled  $\kappa$ B probe and assayed by EMSA. A control of the reaction mix without nuclear extract is included (probe).



**Fig. 3.** Time course of NF- $\kappa$ B activation in HUVEC treated with Daudi cells and TNF- $\alpha$ . Nuclear extracts from untreated (UN) HUVEC, co-cultured for 1, 3 and 6 h with Daudi cells (A) or treated for 0.5, 1, 2 and 3 h with TNF- $\alpha$  (10 ng ml<sup>-1</sup>) (B) were analyzed by western blot analysis using anti-sera to p65 and TFIIB. Results are representative of three independent experiments.

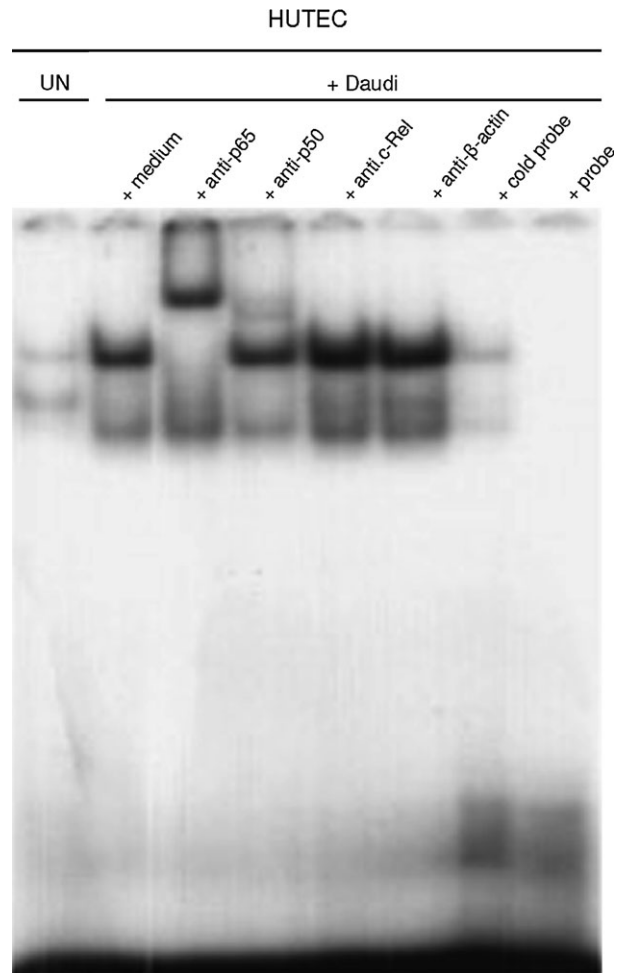
*The NF- $\kappa$ B complex activated by lymphocytes in HUVEC is composed of p65/p50 heterodimers and p50 homodimers*

To determine which of the NF- $\kappa$ B family proteins make up the protein complexes detected by EMSA in nuclear extracts of HUVEC stimulated with Daudi cells, we performed supershift analysis using specific antibodies for p65, p50 and c-Rel. As shown in Fig. 4, anti-p65 antibody caused supershift in the upper complex without affecting the lower complex, while anti-p50 antibody caused supershift in both upper and lower complexes. Anti-c-Rel did not alter the formation of either complex. The use of an irrelevant antibody (anti- $\beta$ -actin) or normal rabbit serum (data not shown) did not affect complex formation. Competition assays using an excess of unlabeled probe showed a decrease in NF- $\kappa$ B binding activity, demonstrating the specificity of complex formation between DNA-binding proteins and  $\kappa$ B nucleotide.

These results indicate that p65/p50 heterodimers formed the upper complex while p50 homodimers formed the lower complex.

*Co-incubation with Daudi cells induces cytokine mRNA and cell-surface adhesion molecule expression in HUVEC*

In order to determine whether NF- $\kappa$ B activation induced by Daudi cells resulted in the functional activation of HUVEC, we studied the production of IL-6 and IL-8 mRNAs by reverse transcription-PCR, and ICAM-1 surface expression by flow cytometry. HUVEC co-cultured with Daudi cells for 3 h expressed higher levels of IL-6 and IL-8 mRNAs in comparison to untreated cells (Fig. 5A). We could not detect significant levels of mRNA for any of these cytokines in Daudi cells recovered from co-culture, thus ruling out the possibility that the increased expression of IL-6 and IL-8 mRNAs detected in HUVEC could come from contaminating Daudi cells. In addition, incubation with Daudi cells for 72 h induced HUVEC

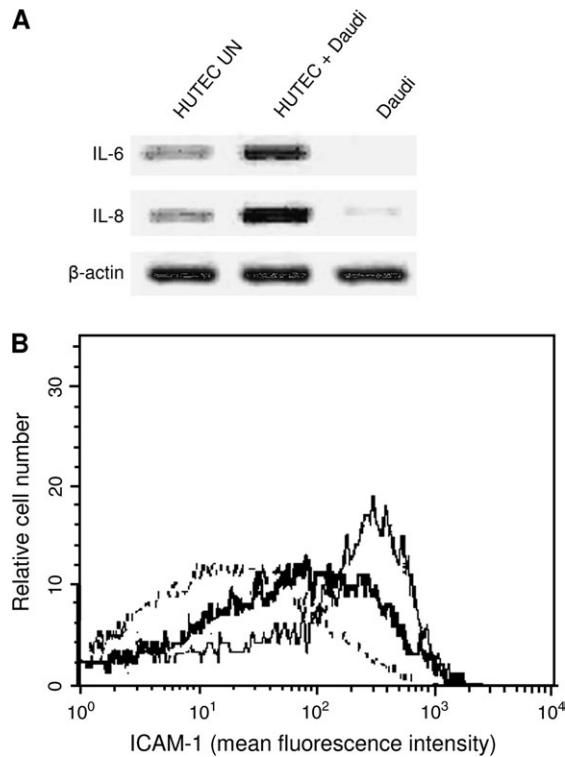


**Fig. 4.** Subunit composition of activated NF- $\kappa$ B in HUVEC co-cultured with Daudi cells. Supershift analysis performed with nuclear extracts from untreated (UN) HUVEC or from HUVEC co-cultured with Daudi cells for 3 h. Before the incubation with <sup>32</sup>P-labeled  $\kappa$ B probe, nuclear extracts were pre-incubated with medium, antibodies specific for p65 (anti-p65), p50 (anti-p50) or c-Rel (anti-c-Rel) proteins, an irrelevant antibody (anti- $\beta$ -actin) or an excess of unlabeled probe (cold probe). A control of the reaction mix without nuclear extract was included (probe).

to express the ICAM-1 (Fig. 5B). These results indicate that interaction of B lymphoid cells with HUVEC results in the increase of markers indicative of the functional activation of the endothelial cells.

*HUVEC activation by Daudi cells requires NF- $\kappa$ B induction*

It has been reported that the non-steroidal anti-inflammatory drug NaSal inhibits NF- $\kappa$ B activation in a variety of cell types (30, 31). We examined the levels of p65 protein in nuclear extracts of HUVEC pre-treated with NaSal for 1 h before their co-culturing with Daudi cells for 3 h. Figure 6(A) shows a dose-dependent inhibition of NF- $\kappa$ B activation by NaSal without affecting the levels of TFIIB proteins. Pre-treatment with NaSal also inhibited, in a similar dose-dependent manner, the induction observed in IL-6 and IL-8 mRNA levels (Fig. 6B). However, NaSal alone did not affect the constitutive



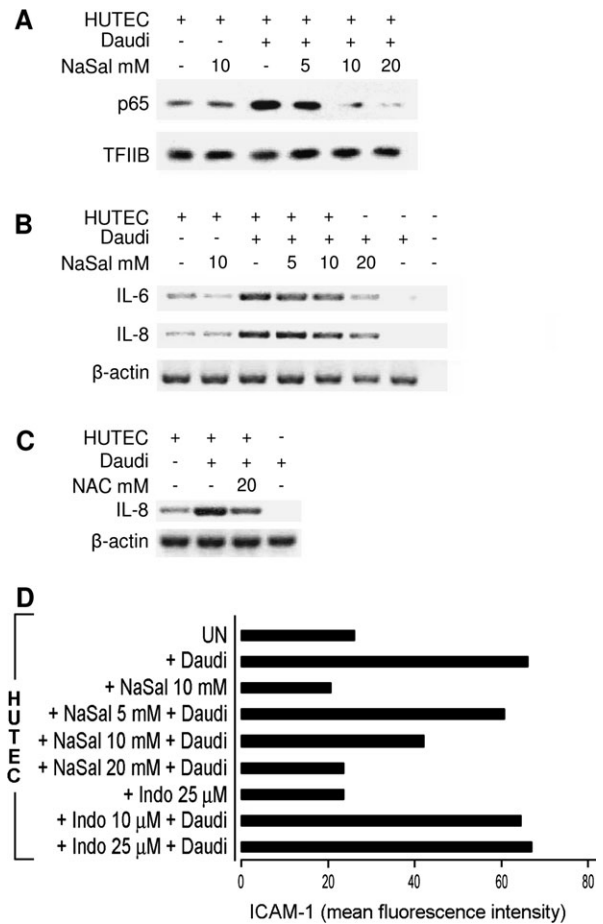
**Fig. 5.** Increased expression of IL-6 and IL-8 mRNAs and ICAM-1 surface expression in HUVEC co-cultured with Daudi cells. (A) mRNA expression levels of IL-6, IL-8 and  $\beta$ -actin assessed by reverse transcription-PCR in total RNA extracted from untreated HUVEC (HUVEC UN), HUVEC co-cultured with Daudi cells (HUVEC + Daudi) for 3 h and Daudi cells recovered from co-culture with HUVEC (Daudi). (B) Cell-surface ICAM-1 expression determined by flow cytometry of HUVEC stained with FITC-conjugated anti-ICAM-1 mAb. HUVECs were left untreated (dotted line), co-cultured with Daudi cells for 72 h (black line) or treated with TNF- $\alpha$  ( $10 \text{ ng ml}^{-1}$ ) for 72 h (gray line). Controls included staining with an isotype-matched Ig (data not shown).

expression of IL-6, IL-8 and  $\beta$ -actin mRNAs, and caused no effect on cell viability (data not shown), indicating that salicylate was not toxic to the cells and did not interfere with transcription or cell function.

The antioxidant NAC, another NF- $\kappa$ B inhibitor, also significantly inhibited the induction of IL-6 mRNA (data not shown) and IL-8 mRNA expression (Fig. 6C). A dose-dependent reduction of the surface expression of ICAM-1 was observed upon pre-treatment of HUVEC with NaSal (5–20 mM) (Fig. 6D). On the other hand, the non-salicylate cyclooxygenase inhibitor, indomethacin (10 or 25  $\mu$ M), had no effect on the levels of ICAM-1 expression (Fig. 6D), indicating that NaSal inhibition does not depend on inhibition of prostaglandin synthesis.

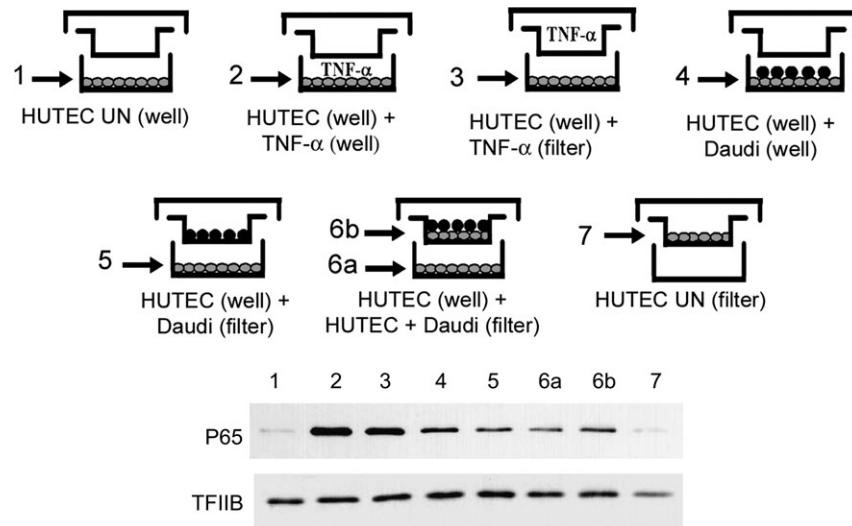
*Is direct contact of Daudi cells or the release of soluble factors responsible for mediating the NF- $\kappa$ B activation in HUVEC?*

We have recently reported that B cell adhesion to HUVEC triggers cell-signaling cascades in the endothelium (20). To assess whether NF- $\kappa$ B activation occurred by direct adhesion or through soluble factors, we co-cultured HUVEC with Daudi cells in Transwell chambers (Fig. 7). HUVECs cultured on the



**Fig. 6.** Inhibitors of NF- $\kappa$ B pathway suppress NF- $\kappa$ B activation, cytokine mRNA and cell-surface ICAM-1 expression in HUVEC co-cultured with Daudi cells. (A) Western blot detection of p65 and TFIIB in nuclear extracts from untreated HUVEC (lane 1), treated with 10 mM of NaSal for 4 h (lane 2) and HUVEC with or without pre-treatment with the indicated concentrations of NaSal for 1 h followed by co-culturing with Daudi cells for 3 h (lanes 3–6). (B) Expression of IL-6 and IL-8 mRNAs assessed by reverse transcription (RT)-PCR of total RNAs from the same cells. Daudi cells recovered from the co-cultures with HUVEC (lane 7) and the reaction mix without cDNA (lane 8) was included as control. (C) RT-PCR analysis for IL-8 mRNA expression on total RNA prepared from untreated HUVEC (lane 1), from HUVEC with or without pre-treatment with 20 mM of NAC followed by co-culturing with Daudi cells for 3 h (lanes 2 and 3) or from Daudi cells recovered from the co-cultures with HUVEC (lane 4). In (B) and (C), we included RT-PCR of  $\beta$ -actin mRNA expression as control. (D) ICAM-1 expression assessed by flow cytometry in untreated (UN) HUVEC, co-cultured with Daudi cells for 12 h (HUVEC + Daudi) or pre-treated with NaSal (HUVEC + NaSal) or indomethacin (HUVEC + Indo) with the indicated concentrations for a period of 1 h and then left alone in culture or co-cultured with Daudi cells during 12 h in the presence of the drugs. Isotype control Ig was included as control (data not shown). Data are presented as mean fluorescence intensity.

bottom well were activated by TNF- $\alpha$  added either in the same well (lane 2) or in the upper chamber (lane 3), demonstrating that this factor readily crosses the polycarbonate filter. As expected from our previous observations, co-culturing Daudi and HUVEC in the same compartment induced p65 in the endothelial cells (lane 4). Unexpectedly, we also detected an increased level of nuclear p65 (lane 5), compared with



**Fig. 7.** Activation of NF-κB in HUVEC co-cultured with Daudi cells is mediated by soluble factors. Top panel, HUVEC (gray circles) grown in the lower wells (conditions 1–6) or upper wells (conditions 6b and 7) of Transwell chambers were left untreated (UN) (conditions 1 and 7), incubated with  $10 \text{ ng ml}^{-1}$  of TNF- $\alpha$  added into the lower (condition 2) or upper (condition 3) compartment for 0.5 h or co-cultured with Daudi cells (black circles) added into the lower (condition 4) or upper (conditions 5 and 6b) compartment for 3 h. Lower panel, Immunoblot detection of p65 and TFIIIB in nuclear extracts from HUVEC incubated in the different conditions described above.

untreated HUVEC (lane 1), when HUVEC and Daudi cells were co-cultured in separate compartments (condition 5). This strongly suggests that NF-κB activation in HUVEC was induced by soluble factors. To confirm that direct cell–cell contact was not necessary, we devised another experiment (condition 6) in which we could determine the effect of direct contact as well as that of soluble factors in a single well. As seen under conditions 6a and 6b, endothelial cells, either without contact (lane 6a) or in direct contact (lane 6b), showed NF-κB activation as detected by nuclear p65. These results suggest that NF-κB activation in HUVEC, induced by interaction with Daudi cells, is mostly mediated by soluble factors. More importantly, a supernatant obtained from a 72-h culture of Daudi cells alone or conditioned media obtained from 72-h HUVEC/Daudi co-cultures did not activate NF-κB when added to HUVEC in culture (data not shown). Thus, these results suggest that B lymphoid cells do not secrete this (putative) activating factor when cultured alone but that the release of soluble factors by lymphoid cells require a cross talk between these cells and HUVEC.

Since TNF- $\alpha$  and lymphotoxin have been described as potent NF-κB activators, we used a bioassay with a sensitivity between 1 and 5 pg of bioactive protein to determine the level of these cytokines in culture media obtained from HUVEC co-cultured with the different lymphoid cells for 3–6 h. No bioactive protein could be detected under these conditions. However, the assay did detect the constitutive production of these molecules by the TBCL-10 cells (data not shown).

## Discussion

Endothelial activation has been principally characterized in response to inflammatory stimuli and mainly using endothelial cells from non-lymphoid tissue (27, 28), which normally do

not allow lymphocyte transmigration. On the other hand, the process of endothelial activation required for sustaining a continuous re-circulation of lymphocytes through secondary lymphoid organs remains relatively poorly understood. In the present work, we used high endothelial cells isolated from human tonsils as a model system to investigate the effect of leukocyte interaction. We found that incubation with B lymphocytes induces an NF-κB-dependent activation of HUVEC. The mechanism involves soluble factors induced through cross talk signaling between both cell types.

NF-κB is a ubiquitous transcription factor extensively employed by cells of the immune system. Its rapid activation in response to a wide variety of signals and its versatility in providing different levels of control are specially suited to the fine-tuning of inducible gene expression required by the immune system. Thus, the NF-κB regulatory system participates in the activation of a great variety of genes involved in the immune response (13, 32), as well as in the endothelial expression of adhesion molecules (12–15) and cytokines (16–18) which are crucial for lymphocyte transmigration. In spite of the known heterogeneity of endothelial cells (33–36), this transcriptional regulatory system has been studied almost exclusively on model systems provided by human umbilical vein endothelial cells or aortic endothelial cells, while its role in the endothelium derived from lymphoid organs, which specializes in lymphocyte transmigration, has remained relatively unexplored (37). We have previously reported that lymphoid cell adhesion induces a differential pattern of cytokine expression in high endothelial cells (20). In these studies, the B cell line Daudi induced the expression of IL-6, IL-8, MCP-1, macrophage colony-stimulating factor and monocyte inflammatory protein-1 $\beta$  mRNA in HUVEC whereas T lymphoid cells had no such effect. In support of these data, the results

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presented herein show that allogeneic tonsillar B lymphocytes and B cell lines (Daudi and TBCL-10 cells) induce a strong NF- $\kappa$ B activation. The effect seen with B lymphocytes could not be due to the low contaminating T cells (<8%) since co-incubation with twice the amount of T lymphocytes had no effect. Activation of NF- $\kappa$ B through co-incubation with B cells leads to an increase of IL-6 and IL-8 mRNAs and ICAM-1 surface expression. High doses of salicylates, reported to inhibit the activity of NF- $\kappa$ B in lymphoid cells (30), also inhibited the activation of NF- $\kappa$ B as well as the induction of IL-6 and IL-8 mRNAs and cell-surface ICAM-1 expression in HUTEc generated by being co-cultured with Daudi cells. Thus, tonsil high endothelial cells use an NF- $\kappa$ B-dependent mechanism to regulate the expression of cytokines and adhesion molecules in response to their interactions with B lymphocytes but not with T cells. Because high doses of salicylates have been reported to inhibit adhesion molecule expression and transmigration of neutrophils (31), such a mechanism could be required to maintain endothelial receptiveness to continuous lymphocyte transmigration.

We used Transwell chambers to assess the effects of co-culturing HUTEc and lymphoid cells either in the same compartment, allowing direct cell-cell interactions, or in separate compartments. Even though we could not completely rule out a contribution of direct cell-cell interaction in NF- $\kappa$ B activation, our results show that this effect can be generated by soluble factors. In this regard, it is important to point out that conditioned media obtained from lymphoid cell cultures or from 72-h HUTEc/Daudi co-cultures (data not shown) could not reproduce the activation effects seen in cultures in which both HUTEc and B lymphoid cell lines were present simultaneously. This suggests that the effect of the putative soluble factors on the endothelium requires cross talk between lymphocytes and endothelial cells. Alternatively, this alleged soluble factor might be labile or it may have an activating effect only if delivered directly to the endothelial cell.

Since TNF- $\alpha$  and lymphotoxin- $\alpha$  have been well characterized as a mediator of endothelial cell activation, we determined the level of these cytokines in the supernatants obtained from co-culture media by using a functional assay. We were unable to detect these bioactive proteins in the conditioned medium obtained from co-cultures of Daudi cells with HUTEc. This is in accord with a previous report showing that Daudi cells do not secrete lymphotoxin (29). Instead, we found that TBCL-10 constitutively secretes TNF- $\alpha$  and/or lymphotoxin, and therefore, its effect upon NF- $\kappa$ B activation in HUTEc is most likely mediated by these cytokines. The identification of the factors involved in the cellular cross talk leading to tonsil endothelial activation remains to be elucidated.

Cross talk between different cell types has been previously reported to occur as part of regulatory mechanisms in a variety of processes. For instance, in murine heart microvascular endothelial cells, the inhibitory effects of IL-12 on proliferation, adhesion, angiogenesis and expression of adhesion molecules require contact-independent continuous interaction with lymphocytes (38). Furthermore, the interaction of bone marrow stromal cells with B cell precursors and the contact between thymic epithelial cells with thymocytes are essential for growth, migration through lymphoid organs and differen-

tiation of developing lymphocytes. Recently, it was shown that these interactions are also required for the functional modulation and development of both bone marrow stromal cells (39, 40) and thymic epithelial cells (41, 42). Our results reveal a cross talk between high endothelial cells and B lymphocytes, leading to cytokine secretion and endothelial activation. This observation may not be directly related to B cell trafficking across HEVs, a phenomenon clearly dependent on direct cell contact. Instead, this phenomenon may contribute to the differentiation of high endothelium, or may be necessary for the preservation of the permanent state of activation of lymphoid endothelium. In this regard, Mebius and co-workers have proposed a role for B lymphocytes in the early organization of lymphoid organs, probably acting through chemokine secretion (43, 44), a role that may be extended to the formation and maintenance of high endothelium. Interestingly, this same phenomenon does not occur with T cells. Because this process involves NF- $\kappa$ B transcription factors, it may be of interest to explore the possibility that anti-inflammatory agents, known to inhibit NF- $\kappa$ B activation, could alter lymphocyte migration.

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### **Abbreviations**

|                |                                      |
|----------------|--------------------------------------|
| ATCC           | American Type Culture Collection     |
| dNTP           | deoxynucleoside triphosphate         |
| DTT            | dithiothreitol                       |
| EMSA           | electrophoretic mobility shift assay |
| HEV            | high endothelial venules             |
| HUTEc          | human tonsil endothelial cell        |
| ICAM-1         | intercellular adhesion molecule-1    |
| MCP-1          | monocyte chemoattractant protein-1   |
| NAC            | <i>N</i> -acetyl-L-cysteine          |
| NaSal          | sodium salicylate                    |
| NF- $\kappa$ B | nuclear factor $\kappa$ B            |
| PMSF           | phenylmethylsulfonyl fluoride        |
| TBCL-10        | tonsil B-cell-derived lymphocyte-10  |
| TFIIIB         | transcription factor IIIB            |
| TNF- $\alpha$  | tumor necrosis factor-alpha          |
| ToL            | tonsillar lymphocytes                |

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