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NFκB is an Unexpected Major Mediator of Interleukin-15 Signaling in Cerebral Endothelia

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Key Words
IL-15 • NFκB • Cytokine receptors • BBB • Endothelial cells • TNF

Abstract
Interleukin (IL)-15 and its receptors are induced by tumor necrosis factor α (TNF) in the cerebral endothelial cells composing the blood-brain barrier, but it is not yet clear how IL-15 modulates endothelial function. Contrary to the known induction of JAK/STAT3 signaling, here we found that nuclear factor (NF)-κB is mainly responsible for IL-15 actions on primary brain microvessel endothelial cells and cerebral endothelial cell lines. IL-15-induced transactivation of an NFκB luciferase reporter resulted in phosphorylation and degradation of the inhibitory subunit IκB that was followed by phosphorylation and nuclear translocation of the p65 subunit of NFκB. An IκB kinase inhibitor Bay 11-7082 only partially inhibited IL-15-induced NFκB luciferase activity. The effect of IL-15 was mediated by its specific receptor IL-15Rα, since endothelia from IL-15Rα knockout mice showed delayed nuclear translocation of p65, whereas those from knockout mice lacking a co-receptor IL-2Rγ did not show such changes. At the mRNA level, IL-15 and TNF showed similar effects in decreasing the tight junction protein claudin-2 and increasing the p65 subunit of NFκB but exerted different regulation on caveolin-1 and vimentin. Taken together, NFκB is a major signal transducer by which IL-15 affects cellular permeability, endocytosis, and intracellular trafficking at the level of the blood-brain barrier.

Introduction

Interleukin (IL)-15 is a ubiquitously expressed 14 kD cytokine. It binds to its unique receptor IL-15Rα as well as two co-receptors IL-2Rβ (also termed IL-15Rβ) and IL-2Rγ common chain. This heterotrimeric receptor complex is the high affinity form that activates Janus kinase (JAK) and Signal Transducer and Activator for Transcription (STAT) proteins [1-6]. In most cells the actions of IL-15 are mediated through the JAK/STAT pathway; however, little is known about IL-15-activated signaling in the microvascular endothelial cells that compose the blood-brain barrier (BBB).

Blood IL-15 levels are low in the resting state but increase after inflammatory and autoimmune challenges.

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In endothelia of mouse cerebral microvessels and cultured rat brain endothelial (RBE)-4 endothelial cells, tumor necrosis factor α (TNF) stimulates both IL-15 and IL-15 receptor expression [7]. Similarly, the mRNA of all three IL-15 receptor subunits (IL-15Rα, IL-2Rβ, IL-2Rγ) in both cerebral microvessels and CNS parenchyma can be increased by lipopolysaccharide (LPS), a prototypic inflammatory stimulus that activates TNF and other proinflammatory cytokines, as well as by experimental autoimmune encephalomyelopathy (EAE) [8, 9]. In mice with EAE, IL-15 treatment provides neuroprotection and reduces behavioral deficits. Thus, we further hypothesize that IL-15 signaling in endothelial cells exerts cytoprotective effects against inflammation and impaired function of the BBB.

The master regulator of gene expression in inflammation is nuclear factor κB (NFκB/Rel). There are five members of the Rel family that can homo- and/or heterodimerize with each other: p65/RelA, RelB, c-Rel, p50, and p52. Dimerization and nuclear translocation are tightly controlled by a family of inhibitory molecules (IκB) that keep NFκB in the cytoplasm [10]. TNF induces NFκB through a signaling cascade that includes binding to the TNF receptor (TNFR), binding of TNFR associated factor (TRAF)-2, activation of IκB kinase (IKK), phosphorylation and proteasomal degradation of IκB, dimerization and nuclear translocation of NFκB, and activation of gene expression by binding to cognate DNA binding sites [11, 12].

Thus, there are at least two potential models of TNF/IL-15 interactions within cerebral endothelia: (a) signaling crosstalk of TNF-induced NFκB and IL-15-activated STAT3 and STAT5, as shown with a few other classes of receptors [13], or (b) convergence or antagonism of TNF and IL-15 signaling at NFκB. Both novel hypotheses could illustrate important principles in endothelial cell biology.

Materials und Methods

Cells

RBE4 cells of rat brain microvessel endothelial cell origin and hCMEC/D3 cells of human brain microvessel cell origin were both kindly provided by Dr. Pierre-Olivier Couraud (Institut Cochin, Paris, France). The cells were plated onto plates coated with collagen (50μg/ml). RBE4 cells were cultured in αMEM/Ham's F10 (1:1; Invitrogen, Carlsbad, CA) supplemented with 10% FBS (heat-inactivated; Invitrogen), βFGF (1 ng/ml; Invitrogen), and G418 (30 μg/ml; Invitrogen). hCMEC/D3 cells were cultured in EBM-2 media (Lonza, Basel, Switzerland) containing all provided supplements but without heparin.

Primary mouse brain microvessel endothelial cells (PBMEC) were isolated from brain cortices of wild-type or IL-15 receptor knockout (KO) mice following an approved Institutional Animal Care and Use protocol as previously described [14]. This includes 4- 6 week old, IL-15Rα KO and matching B6129SF2/J controls, and IL-2Rγ KO and matching B6 controls, all from Jackson Laboratories (Bar Harbor, ME).

Transfection experiments

RBE4 cells were plated onto a 48-well plate. Twenty-four h later, the STAT3 luciferase reporter (0.1μg/well; kindly provided by Dr. Charles Rosenblum, Merck Research Laboratories, Rahway, NJ), the STAT5 luciferase reporter (0.1μg/well; kindly provided by Dr. Charles V. Clevenger, Northwestern University, Chicago, IL), or the NFκB luciferase reporter (0.1μg/well; Agilent Technologies, Santa Clara, CA) combined with a Renilla luciferase (10ng/well; Promega, Madison, WI) as an internal reference plasmid were transfected into 95% confluent cells by use of lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfection of RBE4 cells with GFP plasmids in similar concentrations showed that about 80-90% of cells were transfected. Twenty-four h after transfection and 12 h of serum-starvation, the cells were treated for 6 h with either IL-15 (Peprotech, Rocky Hill, NJ) or TNF (5 ng/ml), or a combination of both as indicated. To test the role of IKK, p38 mitogen-activated protein kinase (MAPK), and Akt on TNF and IL-15-induced NFκB transactivation, we used the inhibitors Bay 11-7082 (Bay; 10 μM; EMD Chemicals, Gibbstown, NJ) and SB203580 (30 μM; Santa Cruz, Santa Cruz, CA) . Cells transfected with the NFκB luciferase reporter were treated for 6 h along with cytokines, and compared with a DMSO vehicle control studied concomitantly. Luminescence was detected by the Dual luciferase assay (Promega) according to the manufacturer's instructions. STAT3, STAT5, and NFκB luciferase activities were normalized by Renilla control luciferase activity. All groups contained triplicated wells, and each result was at least duplicated.

Immunocytochemistry and Western blot analysis

hCMEC/D3 and PBMEC cells were plated onto 8-well chambered Permanox slides (Thermo Fisher Scientific, Pittsburgh, PA) for immunocytochemistry and confluent cells then were incubated for 5, 15, or 30 min with human IL-15 (5 ng/ml; Peprotech). For immunocytochemistry, cells were fixed with 4% paraformaldehyde and then washed with phosphate-buffered saline (PBS), permeabilized for 30 min in PBS/0.1% Triton X-100, and blocked for 1 h in 10% normal donkey serum in PBS. Fixed cells were incubated overnight at 4°C with mouse anti p65 (1:50; sc-8008; Santa Cruz) diluted in PBS/0.1% Triton X-100/1% BSA. Then cells were washed three times with PBS for 5 min and incubated with Alexa488-conjugated anti-mouse secondary antibody (1:1000; Invitrogen) for 1 h. After a thorough wash, slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescence was captured on either an Olympus FV1000 inverted laser scanning microscope or a Zeiss Axioplan 2 fluorescence microscope connected to a Photometrics CoolSnap HQ CCD camera.

For western blot analysis, hCMEC/D3 cells were plated onto 6-well plates. For control, one well was pretreated for 1 h with mouse anti IL-15 antibody (1:100; sc-8437; Santa Cruz), and other wells were treated with either human TNF (5 ng/ml; Peprotech) or IL-15 for 5 and 15 min. Similar experiments were performed in the presence of goat anti-TNFα antibody (1:100; sc-1351; Santa Cruz) in all wells. Cells were lysed with Cellytic (Sigma) and protein concentration was measured by the BCA method (Pierce). Lysates were electrophoresed on an SDS gel and transfered onto a nitrocellulose membrane. After being blocked with PBS-T/5% milk, membranes were incubated with rabbit anti-pp65 (1:2000; ab31472; Abcam; Cambridge, UK), mouse anti-pIκB (#9246), rabbit anti-pSTAT3 (#9131), or rabbit anti-pSTAT5 (#9359) antibodies (1:2000, all from Cell Signaling, Danvers, MA) overnight at 4°C. After being washed, membranes were incubated with either anti-mouse HRP or anti-rabbit HRP (1:5000; Perkin Elmer, Boston, MA), and proteins were visualized by use of ECL substrate (Amersham). Then, membranes were stripped with Restore stripping buffer (Pierce) and incubated with either mouse anti-p65 (1:500; sc-8008, Santa Cruz), rabbit anti-IκB (1:2000; #9242, Cell signaling), rabbit anti-STAT3 (1:1000; 06-596, Millipore), or rabbit anti-STAT5 antibodies (1:1000; sc-835, Santa Cruz) overnight at 4°C. After additional stripping, the IκB blot was incubated with mouse anti-β-actin antibody (1:10000; A5441, Sigma, St. Louis, MO). Membranes were washed, incubated with secondary antibodies, and proteins were visualized as described above.

qPCR

hCMEC/D3 cells (passage # 31-33) were plated onto 6-well plates and incubated for 6 h with either human TNF (5 ng/ml) or human IL-15 (5 ng/ml). Then, mRNA was isolated by use of the ChargeSwitch kit according to the manufacturer's instructions (Invitrogen). mRNA was reverse transcribed with a Superscript RT kit with Oligo dT primers according to the manufacturer's instructions. cDNA concentrations were measured and 50 ng cDNA was used together with gene-specific primers (Table 1) and SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) for qPCR on an ABI7900T. Induction of gene expression was determined by the ΔΔCT method.

Statistical Analysis

Means were presented with their standard errors. One-way ANOVA was performed to determine overall difference among groups, followed by Tukey's post-hoc test where appropriate.

Results

IL-15 is a robust inducer of NFκB activation

It is usually thought that IL-15 signals through STAT3 and STAT5 whereas TNF activates NFκB as well as many other pathways. We tested the activation by IL-15 of both a STAT3 and a STAT5 luciferase reporter in RBE4 cells. STAT3 luciferase reporter induction by IL-15 (100 ng/ml) was not significant (Fig. 1A). Similarly, endogenous phosphorylation of STAT3 was not induced by IL-15 beyond the basal state in another brain microvessel endothelial cell line, the hCMEC/D3 cells (Fig. 1B). To test the hypothesis that TNF and IL-15 interact

Gene Gene bank accession #	Primer sequence 5´...3´	Amplicon size
Claudin-2 NM_020384	F: CTCCCTGGCCTGCATTATCTC R: CAGTGGTGAGTAGAAGTCCCG	180bp
Vimentin NM_003380	F: CCTTGAACGCAAAGTGGAATC R: GACATGCTGTTCCTGAATCTGAG	106bp
Occludin NM_002538	F: TCAAACCGAATCATTATGCACCA R: AGATGGCAATGCACATCACAA	190bp
ZO-2 AF_177533	F: TCCAATCAAAACGCACAAGCC R: TCGGCATCACTGCCATAACTT	125bp
Caveolin-1 NM_001753	F: ACATCTCTACACCGTTCCCAT R: TGTGTGTCCCTTCTGGTTCTG	205bp
TNFR1 NC_001065	F: CCTGCCAGGAGAAACAGAACAC R: GGGACTGAAGCTTGGGTTTGG	356 bp
TNFR2 NM_001066	F : GCCCCACCAGATCTGTAACGTG R: TGAGGCACCTTGGCTTCTCTC	366bp
p65 NM_001145138	F: CTGATGTGCACCGACAAGTGG R: GTTGAT GGTGCTCAGGGATGAC	353bp
TRAF2 NM_021138	F: AACATTGTCTGCGTCCTGAACC R: CGTTCAGGTAGATACGCAGACACA	343 bp

Table 1. Primers used for quantitative PCR

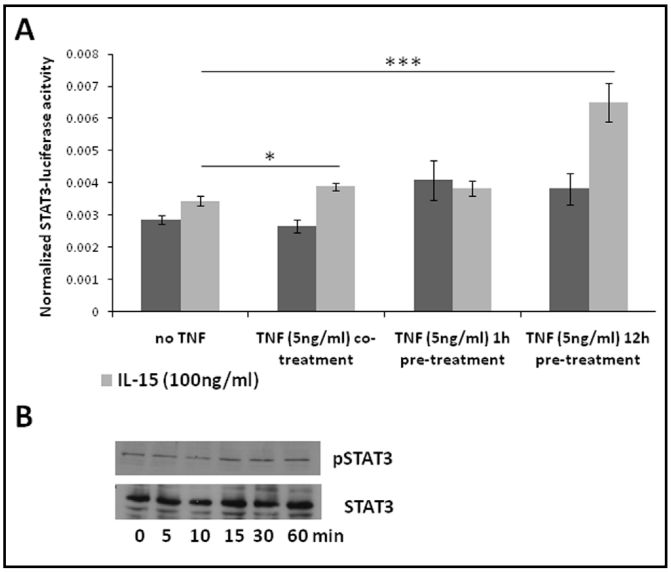
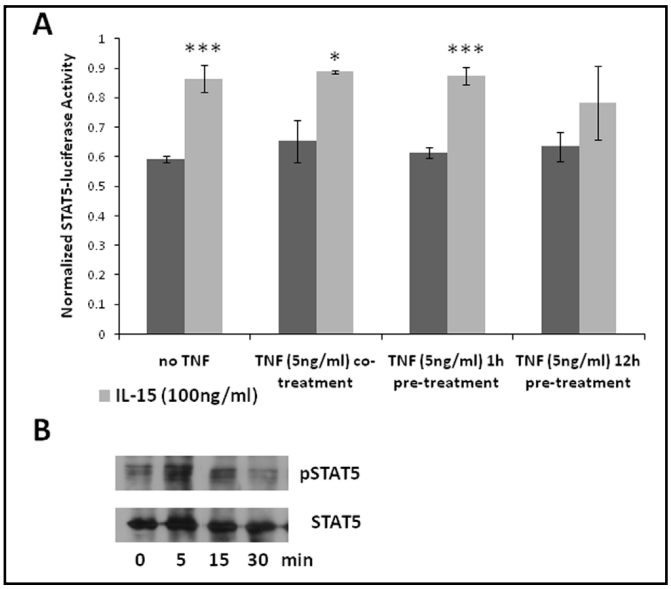


Fig. 1. TNF affects IL-15-induced STAT3 activation. (A) RBE4 cells transfected with a STAT3 luciferase reporter were treated with IL-15 (100 ng/ml; light bars) in the presence and absence of TNF as indicated (dark bars). Co-treatment significantly increased and pretreatment with TNF for 12 h doubled IL-15-induced reporter activity (*$p < 0.05$, ***$p < 0.005$). The graph is representative of three independent experiments, n=3/data point. (B) hCMEC/D3 cells were treated with IL-15 (100ng/ml) for the indicated times and extracts were exposed to western blot analysis. Endogenous STAT3 was not phosphorylated. Similar results were obtained with RBE4 cells. Results shown are representative of each of two independent experiments with hCMEC/D3 and RBE4 cells.

Fig. 2. IL-15 activates STAT5. (A) RBE4 cells transfected with a STAT5 luciferase reporter were treated with IL-15 (100 ng/ml; light bars) in the presence and absence of TNF as indicated (dark bars). Whereas IL-15 induced STAT5 luciferase activity (*p < 0.05, ***p<0.005), TNF had no effect. The graph is representative of two independent experiments, n=3/data point. (B) hCMEC/D3 cells were treated with IL-15 (100 ng/ml) for the indicated times and extracts were exposed to western blot analysis. Endogenous STAT5 was phosphorylated within 5 min. Similar results were obtained with RBE4 cells. Results shown are representative of each two independent experiments with hCMEC/D3 and RBE4 cells.



with each other in STAT3 activation, we performed STAT3 luciferase reporter assays in cells receiving TNF by pre-treatment or co-treatment with IL15. Whereas co-treatment of cells with TNF slightly but significantly increased IL-15-induced STAT3 luciferase reporter activity, pre-treatment for 12 h doubled it (Fig. 1A). However, combined treatment with TNF and IL-15 for 60 min did not increase STAT3 phosphorylation as seen in western blotting (data not shown). Nevertheless, IL-15 increased the STAT5 luciferase reporter 1.5 fold (Fig. 2A), whereas TNF had no effect on reporter activity. Endogenous STAT5 in hCMEC/D3 cells was phosphorylated within 5 min after IL-15 treatment and persisted for 15 min (Fig. 2B).

Unexpectedly, IL-15 alone was sufficient to induce NFκB transcriptional activation in RBE4 cells as shown by the NFκB luciferase reporter assay. TNF was also effective in inducing NFκB transcriptional activation. We used 5 ng/ml of TNF since higher doses are known to impair cellular ATP production and cause cytotoxicity in RBE4 cells [15]. However, the combination of TNF and IL-15 did not induce a further increase of NFκB luciferase activity (Fig. 3A). Dose-response studies showed that 1 ng/ml of IL-15 exerted maximal effect, and there was no additional increase in the group treated with 5-100 ng/ml of IL-15 (Fig. 3B). Since the NFκB luciferase reporter we used contains only five copies of the κB DNA binding site, IL-15 must activate signaling pathways that result in binding of NFκB to its DNA binding site. Therefore, we next tested whether IL-15 induces nuclear translocation of p65, which showed cytoplasmic distribution in hCMEC/D3 cells pretreated with a neutralizing anti-IL-15 antibody. Treatment with IL-15 (5 ng/ml), however, resulted in nuclear translocation of p65 within 15 min. This persisted at 30 min after treatment (Fig. 4A).

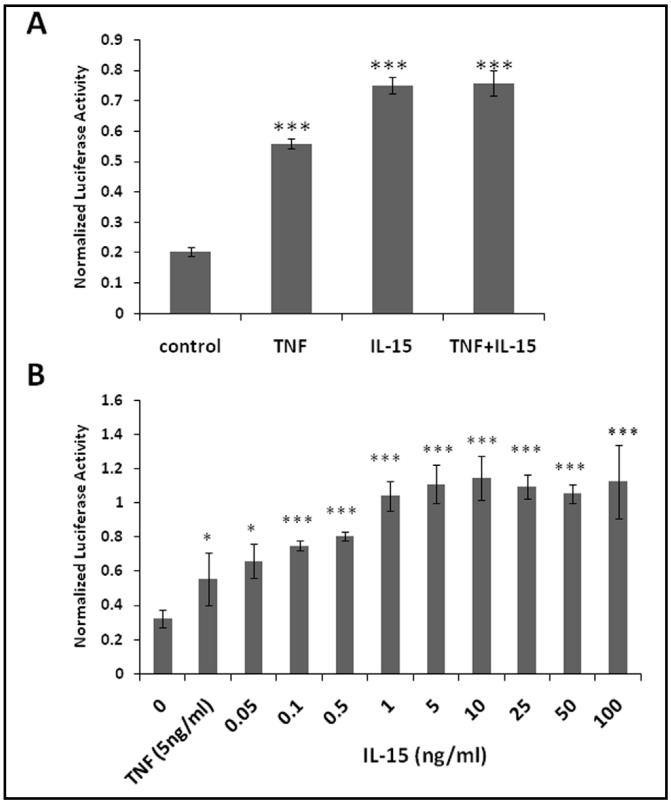
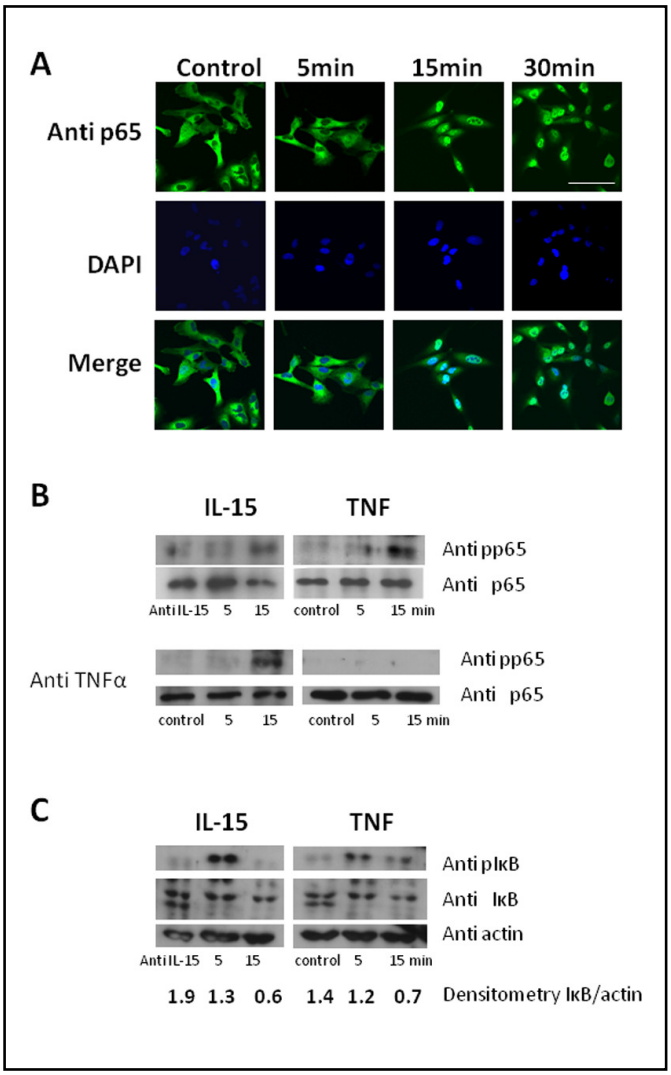


Fig. 3. In RBE4 cells, a low concentration of IL-15 is sufficient for full activation of NFκB luciferase reporter in the absence of TNF. The NFκB luciferase reporter contains 5 copies of the κB DNA binding element (TGG GGA CTT TCC GC). (A) Cells were treated for 6 h with either TNF (5 ng/ml) or IL-15 (100 ng/ml), or a combination of both. The results show that IL-15 activates the reporter (***p<0.005 compared with control). (B) Cells were treated for 6 h with IL-15 only in concentrations as indicated or with TNF (5 ng/ml) for comparison. The results show maximal induction with 1ng/ml IL-15 (*p<0.05, ***p<0.005 compared with control). Graphs are representative of three independent experiments, each with n=3/data point. Data are presented as mean ± S.E.

Fig. 4. IL-15 induces phosphorylation and nuclear translocation of p65 within 15 min after treatment. hCMEC/D3 cells were treated with either IL-15 (5 ng/ml) or TNF (5 ng/ml) for the indicated time. As control, cells were treated with neutralizing TNF (anti TNFα) or IL-15 antibodies (anti IL-15) as indicated. (A) Cells were fixed and stained with an anti-p65 antibody. Bar: 50 μm. Western blot analysis of cell extracts showed (B) phosphorylation of p65 after 15 min with IL-15 and TNF in the absence of neutralizing TNF antibody, but only for IL-15 and not TNF in the presence of neutralizing TNF antibody, and (C) phosphorylation and partial degradation of IκB. Data shown are representative of two independent experiments.



Phosphorylation of p65 typically precedes nuclear translocation. In hCMEC/D3 cells, IL-15 induced phosphorylation of p65 within 15 min, as did TNF (Fig.4B, upper panel). TNF-induced but not IL-15-induced phosphorylation of p65 was abolished in the presence of a TNF neutralizing antibody, indicating the direct activation of p65 by IL-15 (Fig. 4B, lower panel).

TNF-induced activation of NFκB is preceded by phosphorylation and degradation of IκB. To determine whether IL-15 uses a pathway similar to TNF for activation of NFκB, we next determined expression of phosphorylated IκB in hCMEC/D3 extracts. As shown in Fig. 4C, both IL-15 and TNF induced phosphorylation and partial degradation of IκB within 5 and 15 min, respectively.

IκB is mainly phosphorylated by IKK but p38 MAPK and Akt also may play a role in IL-15 mediated activation of NFκB. Therefore, we performed transactivation assays in the presence of inhibitors. Treatment with the IKK inhibitor Bay increased basal transcriptional activity by 45%. As expected, Bay significantly inhibited TNF-induced NFκB activation by 74%, whereas IL-15-induced NFκB activation was only inhibited by 25% (Fig. 5). At the concentration used, SB203580 inhibited both p38 MAPK and Akt. Inhibitor treatment increased basal transcriptional activity by 40%. Whereas SB203580 had no significant effect on TNF-induced NFκB activation (-9%), it inhibited IL-15-induced NFκB activation by 48%. Taken together, these results indicate that IL-15 uses pathways downstream of IκB phosphorylation similar to TNF, whereas upstream pathways may differ.

Role of the specific receptor IL-15Rα

IL-15 activates signaling through its receptors IL-15Rα, IL-2Rβ, and IL-2Rγ, with IL-15Rα being the high affinity receptor, and IL-2Rβ/IL-2Rγ acting as low

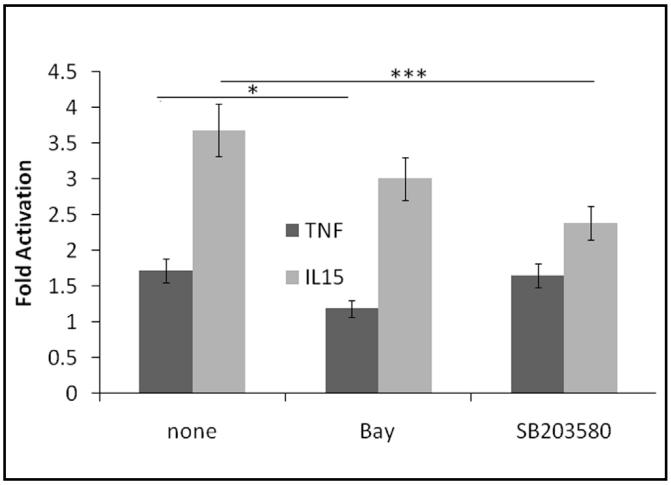


Fig. 5. IL-15-mediated activation of NFκB may involve p38MAPK/Akt pathways. RBE4 cells expressing an NFκB luciferase reporter were pretreated for 1 h with either Bay 11-7082 (Bay) or SB203580. Cells were then treated with either IL-15 (5 ng/ml) or TNF (5 ng/ml) for 6 h in the presence or absence of inhibitors. Data are presented as fold-induction of respective controls. *p < 0.05, ***p < 0.005.

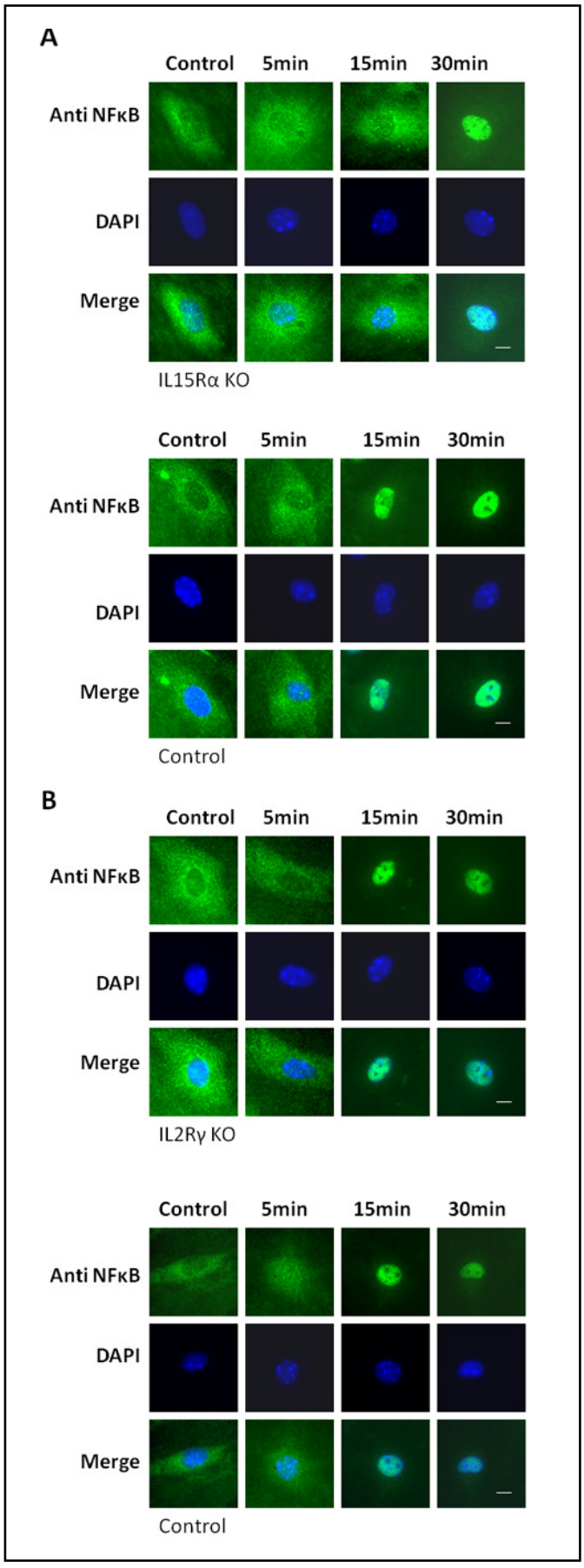


Fig. 6. IL-15-mediated nuclear translocation of p65 is delayed in the absence of IL-15Rα. Primary brain microvessel endothelial cells isolated from mice with genetic knockout of either IL-15Rα (A) or IL-2Rγ (B; upper panels) and their age-matched controls (lower panels) were treated with IL-15 (5 ng/ml) for the indicated time, and then fixed and stained with an anti p65 antibody. Whereas p65 in cells from control and IL-2Rγ KO mice translocated into the nucleus within 15 min, translocation was delayed in cells from IL-15Rα KO mice. Bar: 5μm.

affinity receptors. Most signaling requires the IL-2Rβ/IL-2Rγ dimer. To explore which receptor units are required for activation of NFκB, we used primary microvessel endothelial cells from mice genetically modified to lack either IL-15Rα or IL-2Rγ and their age-matched controls. Treatment with TNF and IL-15 for the indicated time confirmed that p65 translocates into the nucleus of endothelial cells from wild-type mice within 15 minutes, similar to that seen in hCMEC/D3 cells (Figs. 6A and B). Whereas p65 was found in nuclei of cells lacking IL-2Rγ within 15 minutes (Fig. 6B), p65 translocation took 30 minutes in cells lacking IL-15Rα (Fig. 6A). These results indicate that IL-15Rα plays a role in IL-15-mediated NFκB activation.

Effects of IL-15-induced NFκB activation on endothelial functions

To determine how IL-15 modulates endothelial functions, hCMEC/D3 cells were subjected to IL-15 treatment for 6 h and the gene expression profile related to NFκB activation was analyzed by qPCR. Besides the PBS vehicle control, an additional group of cells received TNF treatment for comparison with IL-15. Since TNF increases the expression of IL-15 and IL-15Rα which may in turn regulate genes required for endothelial TNF signaling, we first measured expression of the *p65* subunit of NFκB, *TNFR1, TNFR2,* and the TNFR associated factor 2 (*TRAF2*) known to also bind IL-15Rα [16]. Among these genes, p65 and TNFR2 promoters have NFκB binding sites and TRAF2 is regulated by NFκB [17]. We found that both TNF and IL-15 up-regulate *p65* expression (Fig. 7, upper panel). Both TNF and IL-15 decreased *TNFR1* and increased *TNFR2* expression, but the effects were not significant. Whereas TNF had no effect, IL-15 tended to increase *TRAF2* expression.

Next, we determined the levels of expression of a set of genes related to endothelial functions, including maintenance of BBB properties. This list includes junction-associated proteins and transport proteins. Among them,

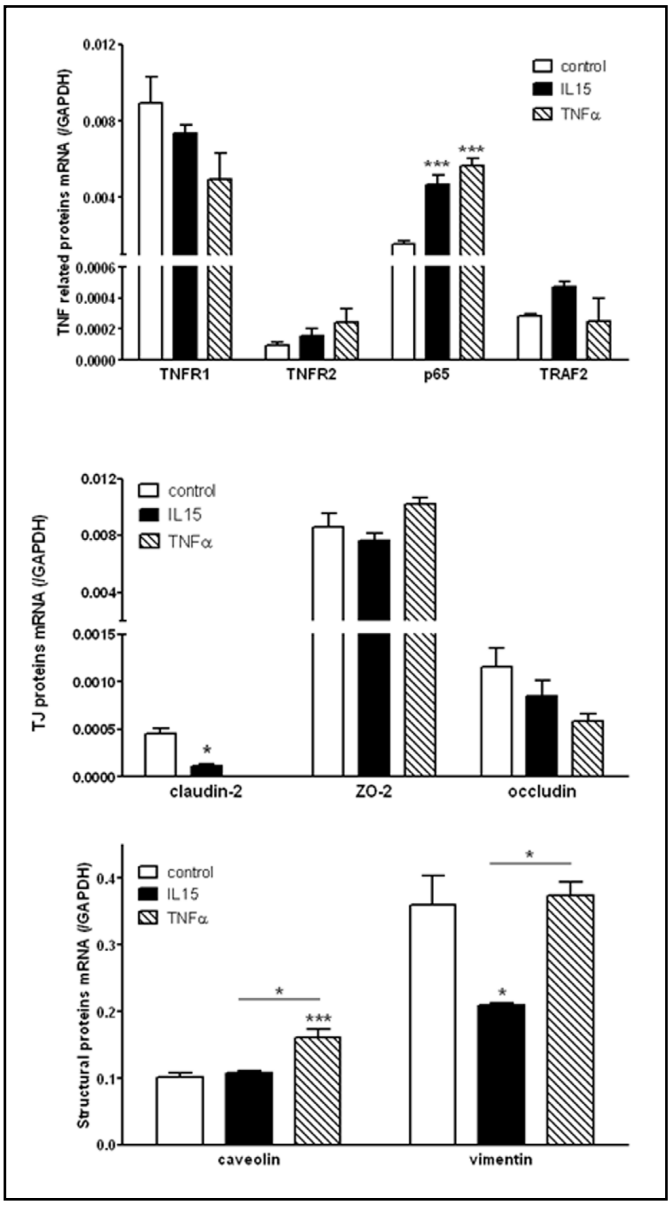


Fig. 7. IL-15 selectively regulates expression of NFκB target genes. hCMEC/D3 cells were treated for 6 h with either IL-15 (5 ng/ml) or TNF (5 ng/ml). qPCR was used to compare the effect of IL-15 and TNF on expression of TNF-related (upper panel), tight junction (TJ; center panel), and structural proteins (lower panel). *Claudin-2, p65, TNFR2, TRAF2, caveolin*, and *vimentin* are potential NFκB target genes, whereas *ZO-2* and *occludin* are not. Graphs are representative of two independent experiments, each with n=3/data point. *p < 0.05, ***p < 0.005.

caveolin [18], claudin-2 [19], and vimentin [20] expression is regulated by NFκB, whereas occludin and zona occludin (ZO)-2 expression is not. We found that IL-15 significantly decreased and TNF abolished the expression of *claudin-2,* whereas neither significantly affected expression of *occludin* or *ZO-2* (Fig. 7, center panel). However, expression of *caveolin* was only affected by TNF, whereas expression of *vimentin* was only affected by IL-15 (Fig. 7, lower panel).

Western blot analysis of p65, TRAF2, caveolin, and vimentin expression in hCMEC/D3 cells after 6, 12, and 24 h treatment with either IL-15 or TNF did not show significant changes in protein expression (data not shown). Taken together, these results indicate that IL-15 plays a role in transcriptional control of p65 and in selective regulation of NFκB target genes.

Discussion

We show here that IL-15 activates an NFκB luciferase reporter, and regulates the transcription of p65 as well as of junction-associated and cytoskeletal proteins in brain microvessel endothelial cells. IL-15 is a powerful antagonist of TNF's pro-apoptotic functions since IL-15 deflects TRAF2 from the TNF receptor toward the IL-15Rα chain [21]. By contrast, we showed that IL-15 induced NFκB activation and nuclear translocation. The results demonstrate that IL-15 induces NFκB target genes both similarly and differently from TNF.

Our results indicate that IL-15Rα plays a role in IL-15-induced NFκB activation. IL-15 recruits TRAF2 to IL-15Rα [21], but IL-2Rβ and IL-2Rγ are not involved. Variations in the strengths of the interaction between TRAF2 and IL-15Rα have been shown in two different melanoma cell lines [16]. This may explain why in some cells but not others IL-15 can induce NFκB [22]. There are multiple pathways that can lead to phosphorylation of IκB. In contrast to TNF, IL-15-induced activation of NFκB was only partially inhibited by the IKK inhibitor BAY, indicating a role of alternative kinases for IL-15-mediated phosphorylation of IκB.

NFκB can also be activated through p38 MAPK and Akt-mediated pathways [23, 24]. IL-1β requires Akt to induce NFκB in an IKK- and p38 MAPK-dependent manner [25]. IL-15 could potentially induce p38 MAPK and Akt through scaffolding proteins interacting with either IL-2Rβ or STAT5 [26, 27]. We found that an inhibitor of both p38 MAPK and Akt partially inhibits IL-15-induced NFκB transactivation, indicating a role of kinases other than IKK in IκB phosphorylation. The activation of p38 MAPK and NFκB in cerebral endothelia by IL-15 is similar to that shown in stimulated microglia [28].

NFκB regulates the proinflammatory response in endothelial cells [29]. TNF activates NFκB mainly by TNFR2-mediated pathways, whereas TNF binding to TNFR1 can induce apoptosis. IL-15 is known to protect

various types of cells from TNF-induced apoptosis [30-34]; this may involve activation of NFκB [16, 35]. TNFR2 and TRAF2 play a role in diverting TNF actions from death-receptor mediated apoptosis to NFκB activation [36, 37]. Our findings show that IL-15 increases the expression of *p65* and *TRAF2*, which may be another mechanism contributing to its anti-apoptotic properties. Although IL-2 and IL-15 share common IL-2Rβ/IL-2Rγ receptor units, this effect is specific for IL-15, as IL-2 does not activate NFκB in neutrophils [38].

The BBB is a unique structure composed by specialized endothelial cells joined by tight junctions, underlaid by a continuous basement membrane, and reinforced by other cellular components such as astrocytic endfeet, pericytes, and microglia [39, 40]. The BBB responds to inflammation with increased cytokine production, activation of transport proteins such as caveolin [41], increased permeability, and selective upregulation of cytokine transport [42, 43]. NFκB plays a role in these changes, and this may be mediated by target gene products such as caveolin-1 [18] and the tight junction protein claudin-2 [44]. Beyond inflammatory signaling affecting tight junction proteins, NFκB may also influence endothelial functions by regulating target gene products such as vimentin [20], an intracellular intermediate filament contributing to endothelial cell shape and implicated in formation of invadopodia [45, 46]. Thus, comparison of the effects of TNF and IL-15 on the target genes regulating BBB functions shows that the effects of NFκB activation may be cytokine-specific.

Since NFκB is a transcription factor shared by TNF and IL-15, we determined the target genes in cerebral endothelia that are potential targets of regulation by both cytokines. Among the genes tested, *claudin-2*, known to be regulated by NFκB, was decreased by both TNF and IL-15, but *vimentin* and *caveolin* were differentially regulated. This indicates that transcription factors other than NFκB may play a more important role in the expression of these proteins. For example, the promoter of the *caveolin* gene contains both an NFκB and a STAT5 binding site, so that IL-15 might act through both transcription factors to regulate *caveolin*.

The expression profile of tight junction proteins in hCMEC/D3 cerebral endothelial cells differs from that in intestinal cells in response to IL-15. In our study, IL-15 had no effect on *ZO-2* expression. In the human intestinal epithelial cell line T-84, IL-15 up-regulates ZO-2 independently of IL-2Rβ but facilitates the functions of occludin and claudins by IL-2Rβ [47]. We showed that TNF suppressed *claudin-2* expression in cerebral endothelia; however, TNF acts through NFκB and increases *claudin-2* in HT-29/B6 human colon cells [44]. Nonetheless, the TNF-induced increase of *caveolin* [48] and decrease of *occludin* expression in endothelial cells [49] is consistent with our results. We chose the hCMEC/D3 cell line as a model system since TNF activates NFκB in these cells and they express TNF receptors [50, 51]. Overall, there is cell type specific regulation of junctional proteins.

Another novel finding is that IL-15 decreases *vimentin* expression. Vimentin is an intermediate filament important for the three-dimensional structure of endothelial cells. In astrocytes, vimentin also plays a role in vesicular trafficking [52]. During hypoxia, expression of vimentin increases in brain microvessel endothelial cells [53]. Similar to our results, TNF caused no change in vimentin expression in bovine cerebral microvessel endothelia [54]. Thus, IL-15 may act both as a mediator and an antagonist of TNF actions though it is produced in response to TNF.

An unusual finding is that TNF co-treatment with IL-15 increased activation of the STAT3 but not of the STAT5 luciferase reporter. Since TNF and IL-15 co-treatment did not induce STAT3 phosphorylation within minutes, the increase in STAT3 activation is probably indirect. The effect of TNF on IL-15-mediated activation of STAT3 is most pronounced after 12 h pretreatment, which may be explained by the inducing effect of TNF on IL-15 and IL-2Rγ expression in RBE4 cells [7]. This TNF-mediated increase in STAT3 activation may result in the regulation of genes important for functions of brain microvessel endothelial cells by IL-15.

Taken together, in brain microvessel endothelial cells IL-15 can induce both STAT5 and NFκB. Activation of NFκB appears to be more sensitive to low concentrations of IL-15 and TNF does not have cumulative or additional effects on STAT5 and NFκB activation by IL-15. By contrast, TNF affects IL-15-dependent STAT3 activation. These results indicate a complex but specific crosstalk between TNF- and IL-15-induced signaling in brain microvessel endothelial cells. Endothelial signaling is an integral part of the BBB response to cytokines, and it plays an essential role in modulation of CNS functions. Besides illustrating that NFκB is an unexpected mediator for IL-15 signaling at the level of the BBB endothelia, we showed that IL-15 signaling modifies the actions of TNF. Since upregulation of the IL-15 system is a major inflammatory response of the BBB endothelia to TNF [7], IL-15 signaling appears to provide feedback control for TNF function. This indicates a dynamic regulation of BBB functions.

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