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Research paper

Immune response in the relapsing-remitting experimental autoimmune encephalomyelitis in mice: The role of the NF-κB signaling pathway

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ABSTRACT

Characteristics of the mouse model of relapsing-remitting experimental autoimmune encephalomyelitis (rEAE) closely resemble manifestations of multiple sclerosis in humans. In the present study, we investigated the mechanisms of inflammatory response, focusing on NF-κB pathway activation. Cytokine response in rEAE mice was multiphasic: the early phase was characterized by the increase in interferon-γ level in plasma. In the later stage, the level of interleukin-17, but not of interferon-γ, was increased. The early phase of rEAE was also accompanied by increased RelA/p65 phosphorylation at Ser276 in spleen cells, whereas the rEAE maintenance phase was characterized by RelA/p65 phosphorylation at Ser536 and IKK phosphorylation. The IKKα/β inhibitor reduced interleukin-17 and interferon-γ levels in plasma and alleviated rEAE symptoms. The IKKα/β inhibitor decreased IKK and p65(Ser536) phosphorylation, but doubled p65(Ser276) phosphorylation in rEAE mice. The increased RelA/p65(Ser276) phosphorylation coincided in time with the production of interferon-γ, Hsp72, and the early phase of IL-17 generation, whereas increased RelA/p65(Ser536) phosphorylation coincided with the activation of IKK, SAPK/JNK, and p53, as well as the late phase of IL-17 production, indicating the role of the RelA/p65 phosphorylation events in the induction and maintenance of rEAE.

1. Introduction

Autoimmune diseases are a group of disorders with diverse clinical manifestations caused by a pathological immune response that leads to the production of autoimmune antibodies and proliferation of auto-aggressive clones of killer cells. Multiple sclerosis is one of the most prevalent autoimmune diseases, affecting over one million people worldwide [1]. In patients with multiple sclerosis, auto-aggressive *T*-cells attack myelin components in the brain and spinal cord, inducing central nervous system (CNS) demyelination, which leads to paresthesia, paraparesis, neuritis, and ataxia. Experimental autoimmune encephalomyelitis (EAE) is an inducible inflammatory autoimmune condition in mice that resembles multiple sclerosis in humans [2]. EAE can be induced in different mouse strains using different myelin components. There are several types of EAE that differ in the course of the disease. For example, in C57BL/6 mice, the disease displays a chronic-progressive clinical course, in other mouse strains, such as PL/J or B10.PL, the disease is normally acute, self-limiting, and devoid of clinical relapses [3]. In SJL mice, the disease is characterized by a relapsing-remitting course of paralysis, resembling a similar clinical symptom in multiple sclerosis in humans. This animal model allows the assessment of the efficacy of various immunomodulatory strategies in the progressive autoimmune disease setting, as well as investigation of the mechanisms underlying periodical remissions. The mechanisms of autoimmune responses may be examined by studying the activity of immune cells and their communication through the cytokine network, as well as by monitoring intracellular signaling.

Interleukin-17-producing *T*-helper (Th17) cells and interferon-secreting Th1 cells are discrete populations of inflammatory *T*-cells that have distinct and possibly complementary roles in the pathogenesis of autoimmune diseases and in the protection against infection [4]. Differentiation of Th1 cells is mediated by IL-12, whereas IL-23, especially in synergy with IL-1, plays an essential role in the induction and expansion of murine and human Th17 cells [5–7]. Several studies have demonstrated that Th17 cells are “true” effectors for autoimmune inflammation in the CNS [5,8,9,10]. Our previous data on the time-course of cytokine response during two different acute EAE forms (mild and severe) showed that the activation of Th1 cell population was followed by the activation of Th17 cells during acute EAE development [11,12], which generated a biphasic cytokine response. Currently, there is a lack of data on the time-course of activation of Th1 and Th17 cells during EAE relapses and remissions, although such knowledge is essential for understanding the mechanisms of multiple sclerosis in humans.

At the intracellular level, the NF-κB signaling pathway plays a

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critical pathological role in inflammatory diseases, including autoimmunity. This pathway is well established as a regulator of innate and adaptive mechanisms of host defense, and specific genetic defects that confer immune deficiency confirm the importance of these mechanisms. There is also abundant evidence that the NF-κB pathway is crucial for maintaining immunological tolerance as it participates in the negative selection of autoreactive T cells and in the selection and maintenance of regulatory T cells [13]. Our previous data [11,12] showed that the induction of acute EAE forms was accompanied by NF-κB pathway activation, whereas NF-κB signaling inhibition alleviated acute EAE symptoms.

Currently, it is thought that certain phosphorylation events contribute to the selective regulation of NF-κB pathway transcriptional activity in a gene-specific manner. It is clear that the phosphorylation of NF-κB subunits does not act as a simple on/off switch, but may either enhance or down-regulate the transcription of target genes [14]. The studies of NF-κB phosphorylation have concentrated primarily on the p65 NF-κB subunit, and the bulk of the published information is focused on the two best-characterized phosphorylation targets within this subunit, Ser276 and Ser536. Ser276 of p65 is phosphorylated by protein kinase A (PKA). In resting cells, the PKA catalytic subunit PKA-C is bound in an inactive state to cytosolic IκB-p65 complexes. Following IκB kinase (IKK) complex activation and IκB degradation, active PKA-C is liberated that phosphorylates p65 at Ser276 in a cAMP-independent manner [15,16]. In addition, p65 Ser276 phosphorylation is also mediated by a number of other kinases, such as Raf-1, mitogen- and stress-activated kinase (MSK) 1/2, and RSK p90 [17–19]. Ser276 phosphorylation triggers a conformational change in p65 that promotes its interaction with CBP/p300 and thereby increases p65 transcriptional activity [20]. Furthermore, phosphorylation of S276 by Pim-1 kinase prevents p65 ubiquitination, which enhances p65 half-life and increases its transcriptional activity [21].

Phosphorylation of Ser536 in the transactivation domain of p65 leads to enhanced transactivation through increased CBP/p300 binding and acetylation at K310 of p65 [22]. As with Ser276, a number of kinases have been identified that phosphorylate Ser536, which include IKK, ribosomal subunit S6 kinase 1, IKKβ, IKKε, and NF-κB activating kinase/TANK-binding kinase 1 [23–26]. During T cell activation, IKK phosphorylates Ser536 within the cytoplasmic IκB-p65 complex, which requires prior phosphorylation of IκB by IKK [27]. Studies using transgenic mice that expressed kinase-dead IKK identified an important role for Ser536 phosphorylation in promoting the proteasomal degradation of p65. The latter seems to be an important mechanism in limiting NF-κB transcription responses in toll-like receptor activated macrophages. In macrophages, the phosphorylation of p65 at Ser536 by IKK increases p65 turnover, thereby reducing NF-κB activity and supporting the resolution of inflammation [24]. There are indications that p65 phosphorylated at Ser536 is not associated with, or regulated by, IκB and that Ser536 phosphorylation may trigger the expression of a distinct set of target genes [28]. Thus, Ser536 phosphorylation of p65 may determine the ability of IκB to inhibit transcription in a gene-selective manner. Considering the importance of selective regulation by site-specific phosphorylation of p65, we sought to investigate the role of these mechanisms in autoimmune responses.

2. Materials and methods

2.1. Animals

Female 3-month-old SJL/J mice (purchased from the Breeding Facility for Laboratory Animals, Pushchino, Russia) weighing 20–25 g were kept in standard laboratory conditions (20–22 °C, 10 h:14 h light:dark cycle, 65% humidity) and supplied with food and water *ad libitum*. Standard mouse food pellets contained a balanced diet with proteins, vitamins, and minerals. The procedure was approved by the Institutional Ethical Committee (approval #57, 12/30/2011), and the experiments were performed in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals.

2.2. Induction of EAE and application of an IKK inhibitor

To induce acute EAE, 150 μg of proteolipid protein (PLP) peptide 139–151 (GenScript, Piscataway, NJ, USA) was emulsified with complete Freund's adjuvant containing 4 mg/mL H37Ra (*Mycobacterium tuberculosis*; Chondrex, Redmond, WA, USA). As described previously [4], a single immunization with 3x30 μL of the emulsion was performed subcutaneously into several sites at the base of the tail of SJL/J mice. Immediately after the immunization (day 0) and in 48 h thereafter, 400 ng of pertussis toxin (US Biological, Swampscott, MA, USA) was injected intraperitoneally. Only mice that developed EAE signs were randomized to the experimental groups. Mice were examined every other day for the signs of EAE, and the severity of disease was graded using the following scale: 0, normal; 1, limp tail; 2, wobbly gait; 3, hind limb weakness; 4, hind limb paralysis; 5, tetraparalysis/death. To examine immunological parameters, there were three experimental groups with 20 mice per group: control, EAE, EAE + inhibitor. Animals were killed in subgroups of five on days 12, 18, and 25. The IKK inhibitor XII (Merck, Germany) at a dose of 1.8 mg/kg in 0.2 mL of saline was injected intraperitoneally every other day for the whole observation period (25 days).

2.3. Blood plasma and cells

Blood was collected following decapitation of the animals. Blood samples were kept for 3–5 h at 4 °C and centrifuged at 200 × g to collect plasma cytokine assays. Lymphocytes were isolated from the spleen in medium 199 (Sigma, USA) containing 1% 1 M HEPES solution, 100 μg/mL streptomycin, and 10% fetal bovine serum. Erythrocytes were lysed in Tris-buffered ammonium chloride (0.01 M Tris-HCl with 0.15 M NaCl and 0.83% NH_4Cl at a ratio of 9:1). After washing, the samples were stored at a concentration of 1×10^8 cells/mL in RPMI 1640 medium at −20 °C until ELISA.

2.4. Elisa

ELISA was used to determine plasma cytokine concentrations. ELISA Development Kits for mouse TNF-α, IL-6, IL-10, IL-17, and IFN-γ (PeproTech, USA) were used. To visualize binding, 100 μL of ABTS green dye (Sigma) dissolved in 0.05 M citrate buffer (pH 5.0) with 0.01% hydrogen peroxide was added, and optical density was measured at 405 nm using a microplate spectrophotometer (Multiskan EX; Thermo Electron Corporation, USA).

2.5. Western blot analysis

To prepare specimens, 1×10^8 splenic cells were lysed using an ultrasonic disintegrator (UDZN-2 T; Moscow, Russia) at 30–35 kHz and 50 μA for 2 min with constant stirring. The total protein concentration in each sample was then determined by the Bradford method. Subsequently, the proteins in each sample were precipitated with acetone, solubilized, boiled for 5 min, and stored at −70 °C. The proteins were resolved by electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham/GE Healthcare, UK) in a Trans-Blot chamber (Bio-Rad, USA). After blocking, the membrane was exposed to an antibody against one of the following mouse proteins for 2 h: heat shock protein (Hsp)70 [rabbit anti-Hsp72 antibody, clone SPA-812, inducible form; Enzo, USA; diluted 1:1000], phospho-NF-κB [rabbit anti-phospho-NF-κB p65 (Ser276) antibody; Cell Signaling Technology, USA; diluted 1:1000], phospho-NF-κB [rabbit anti-phospho-NF-κB p65 (Ser536) antibody; Cell Signaling Technology, USA; diluted 1:1000], NF-κB (rabbit anti-NF-κB p65 antibody; Cell Signaling Technology; diluted 1:1000), phospho-IKKα/β [rabbit anti-

phospho-IKKα/β antibody (Ser176/180); Cell Signaling Technology; diluted 1:1000], IKKβ (rabbit anti-IKKβ antibody; Cell Signaling Technology; diluted 1:1000), phospho-SAPK/JNK [rabbit anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody; Cell Signaling Technology; diluted 1:1000], SAPK/JNK (rabbit anti-SAPK/JNK antibody; Cell Signaling Technology; diluted 1:1000), phospho-p53 [rabbit anti-phospho-p53 (Ser46) antibody; Cell Signaling Technology; diluted 1:1000], p53 [rabbit anti-p53 (1C12) antibody; Cell Signaling Technology; diluted 1:1000], caspase 3 [rabbit anti-caspase-3 (8G10) antibody; Cell Signaling Technology; diluted 1:1000]. After washing, the membrane was incubated for 1 h with a biotinylated goat anti-rabbit antibody [Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H + L); Jackson ImmunoResearch, USA; diluted 1:200,000], before being exposed to 0.1 μg/mL peroxidase-conjugated streptavidin (Jackson ImmunoResearch) for 1 h. To control for variations in protein loading, an antibody raised against a synthetic peptide corresponding to an amino acid sequence near the carboxy-terminus of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used [rabbit anti-GAPDH (14C10) monoclonal antibody; Cell Signaling Technology; diluted 1:1000]. ECL Plus chemiluminescence reagents (Amersham/GE Healthcare) and Hyperfilm ECL (Amersham/GE Healthcare) were then used to develop the blots according to the manufacturer's instructions. The developed films were observed with a Vilber Lourmat (France) TFX-35.WL transilluminator. Quantitative evaluation of protein bands was subsequently performed using QAPA software v. 3.7 (Russia).

2.6. Statistical analysis

Statistical analysis was performed using Statistica 6.0 software (StatSoft, USA). One-way analysis of variance, followed by the post-hoc Fisher's Least Significant Difference (LSD) test, was used to determine the significance of differences among the groups, with p-values ≤ 0.05 being considered significant. All values are expressed as the mean ± standard error of the mean.

3. Results

3.1. Progression of relapsing-remitting EAE

The time course for the relapsing-remitting form of EAE (rEAE) induced by immunization with PLP was as follows: first signs of pathology occurred on day 9 after the immunization and the symptoms continuously intensified up to day 14, when the peak mean severity score was achieved (Fig. 1). Thereafter, some alleviation of symptoms in most mice was observed, followed by subsequent relapses and remissions in many mice. Lethality in this model was low, and almost all mice survived until the end of the experiment, despite of severe paralysis. On days 12, 18, and 25, mice were killed in subgroups of five to analyze immunological parameters. The subgroups comprised mice with severity scores nearest to the current mean severity score for each group. The IKK Inhibitor XII injected repeatedly every other day significantly reduced the severity of the disease when compared with the state of untreated rEAE mice, with complete restoration in many cases (Fig. 1).

3.2. Cytokine response

To characterize the time course of cytokine response, serum concentrations of the cytokines IFN-γ, IL-6, IL-17A, IL-10, and TNF-α were measured by ELISA at different stages of rEAE progression. The results (Fig. 2) showed that in the early stage of the disease (day 12 after the immunization), plasma concentrations of IFN-γ and IL-17A were increased by 40–70% when compared to those in healthy controls. However, IL-6, IL-10, and TNF-α levels in blood were unchanged. On day 18, IL-17A and IFN-γ levels normalized, moreover, IL-17A concentration decreased below control level. On day 25, IL-17A levels were again increased, whereas the levels of other cytokines remained similar to those in control group. Therefore, these results indicate a biphasic cytokine response with an early phase characterized by the increase in plasma IL-17 and IFN-γ and a delayed phase characterized by the increase in plasma IL-17A. Thus, our data in the rEAE model in some respects correspond to biphasic cytokine response observed earlier in the acute EAE models, where an initial IFN-γ increase and a subsequent IL-17A increase were observed [11,12].

3.3. NF-κB pathway activity

To investigate intracellular mechanisms of immune cell activation in rEAE, a role for the phosphorylation of different sites on the RelA/p65 protein was studied in spleen lymphocytes from mice in the three experimental groups (Fig. 3). The results showed that in mice with EAE, RelA/p65 phosphorylation at serine 276, which is attributed mainly to PKA, was markedly (fourfold) increased compared to the level in control animals during the earliest stage of the disease (day 12). At the later stages, the phosphorylation at serine 276 decreased down to control levels (Fig. 3A). The treatment with the IKK kinase inhibitor XII not only did not decrease serine 276 phosphorylation, but doubled it compared to the level in the EAE group.

Furthermore, we investigated the phosphorylation of the RelA/p65 protein at serine 536, which is thought to be a target of IKK. Our results (Fig. 3B) showed no changes of Ser536 phosphorylation at the earliest stage of the disease (day 12), but gradual increases in Ser536 phosphorylation levels were observed at the later stages (days 18 and 25). The magnitude of the increase was about 200% on day 25. The IKK inhibitor XII strongly attenuated Ser536 phosphorylation. Therefore, Ser276 phosphorylation and Ser536 phosphorylation apparently proceeded in opposite directions and occurred at different stages of the experiment.

Furthermore, IKK phosphorylation did not occur at the earliest stage (day 12), but was observed at the later stage (day 18) of the experiment, in correspondence with the above mentioned data. At the latest stage (day 25), however, IKK phosphorylation correspondingly decreased to normal values (Fig. 3C).

3.4. Activity of the SAPK/JNK and p53 pathways and heat-shock protein Hsp72 expression in spleen cells

The activation of stress-related pathways in immune cells in rEAE, the levels of phosphorylated forms of stress-activated protein kinase/c-Jun terminal kinase (SAPK/JNK) and p53 as well as the levels of inducible heat-shock protein were assessed by western blotting. Our results showed that the early stage of the disease (day 12) was not accompanied by SAPK/JNK phosphorylation (Fig. 4A). However, this kinase was markedly phosphorylated (and, therefore, activated) at the later period and remained so up to the end of the observation period. The IKK inhibitor did not significantly change SAPK/JNK phosphorylation.

We also measured the levels of phosphorylated and total p53 protein (Fig. 4B). The increased ratio of phospho-p53 to total p53 in spleen lymphocytes indicated that this pro-apoptotic and cell cycle regulating pathway was activated at the latest stage of rEAE only (day 25), but not at the earlier stages. The IKK inhibitor did not significantly change p53 phosphorylation.

The expression of inducible heat-shock protein Hsp72 was elevated at the earliest stage only, decreasing to the control levels at the later stages (Fig. 4C). It should be noted that the IKK inhibitor not only did not block Hsp72 expression, but increased it two-fold compared to the level in the EAE group, similarly to the modulation of RelA/p65 phosphorylation at serine 276.

Therefore, the phosphorylation of the RelA/p65 protein at serine 276 coincided in time with Th1 cytokine production, early Th17 cytokine production, and an increase in the Hsp72 level. In addition, the

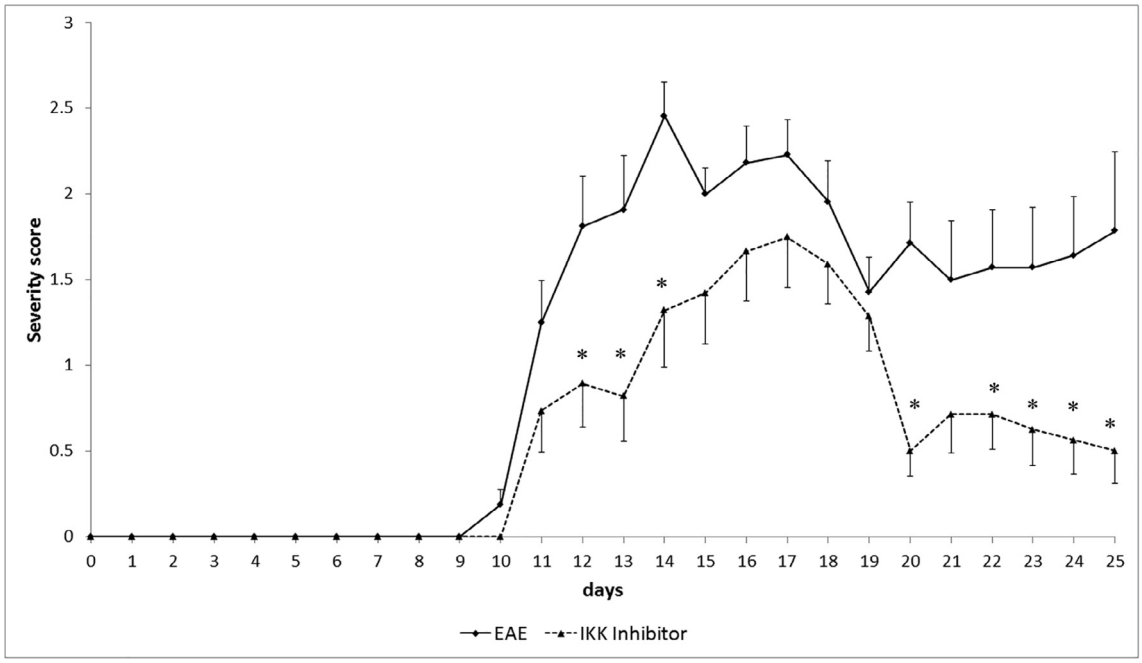

Fig. 1. Severity score in mice with experimental autoimmune encephalomyelitis (EAE). Data from untreated mice (solid line) and mice treated with the IκB kinase inhibitor XII (dotted line) were obtained every other day throughout the observation period. The day of immunization with proteolipid protein peptide is indicated as day 0. Data are presented as the mean severity score ± standard error of the mean for 10–20 mice per group, depending on the time period. Clinical signs of EAE were recorded every day. *Significantly different from untreated mice, $p < 0.05$.

phosphorylation of the RelA/p65 protein at serine 536 coincided with the activation of the IKK, SAPK/JNK, and p53 pathways, and Th17 cytokine production at the later stage.

4. Discussion

Our data demonstrated that the rEAE model exhibited rather mild pathology with low mortality rate. Furthermore, the severity of neurological symptoms was intermittently increased and decreased. Such a progression was similar to a typical course of multiple sclerosis in humans, which is often characterized by intermittent relapses and remissions. Our time-course findings were consistent with numerous previously reported data in this model [4]. Such features make rEAE most corresponding to multiple sclerosis in humans. Previously, we investigated two other EAE models with progressively increasing symptoms that were characterized by mild or severe progression (mEAE and sEAE, respectively), which enabled comparisons of autoimmune responses and, in some cases, effects of therapy.

In the present work, we observed a biphasic cytokine response, whereby the earlier phase was characterized by an increase in interferon-γ, whereas the later phase was associated with an increase in IL-17, along with the restoration of interferon-γ to the normal level. It was generally consistent with our previous results in the models of mild and severe EAE [11,12], supporting our hypothesis about sequential activation of immune cell populations in autoimmune inflammation. Furthermore, IL-17 level was elevated on days 12 and 25, but not on day 18 in the rEAE model. Thus, we observed a biphasic IL-17 profile in rEAE, in contrast to its profile in progressive forms of EAE. In addition, the levels of general pro-inflammatory cytokines, such as IL-6 and TNF-α, in rEAE were similar to those in control, whereas in the progressive forms, they were elevated to a greater or lesser extent. Therefore, the relapsing remitting autoimmune process had some differences in cytokine profile from that of continuously progressing EAE forms, but IFN-γ- and IL-17-producing cell subpopulations had a key role in all of these responses. These results may help to clarify the roles of certain immune cell subpopulations in EAE and multiple sclerosis. It is known that patients with multiple sclerosis exhibit various forms of disease with different immunopathologies. These clinical forms are caused by different subsets of $CD4^+$ T helper (Th) cells with predominant generation of either IL-17, the hallmark of Th17 cells, or IFN-γ, the hallmark of Th1 cells [29]. It has been shown that different forms of EAE are characterized by different profiles of IFN-γ and IL-17 [30], and that Th1 and Th17 cells have a central role in disease development [31–33].

Our data also indicate that Th1-response was IKK-dependent, because the IKKα/β inhibitor completely blocked IFN-γ increase in plasma of EAE mice. In contrast, IL-17 response was not blocked by this inhibitor, indicating a different mechanism of its activation. These results are also supported by our data in the severe EAE model, when similar results were obtained using the IKKα/β inhibitor [12]. To investigate intracellular mechanisms of immune cell activation, we examined phosphorylation of several signaling proteins of the NF-κB cascade, SAPK/JNK cascade, p53 protein, and Hsp72 protein in mice with rEAE.

NF-κB is a well-established intracellular regulator of innate and adaptive mechanisms of host defense that regulates a wide variety of pro-inflammatory genes. In unstimulated cells, NF-κB family proteins (i.e., RelA(p65), RelB, c-Rel, NF-κB1, and NF-κB2) exist as hetero- or homo-dimers with an inhibitory protein complex from the IκB family. The phosphorylation and degradation of inhibitory IκB proteins through the action of specific kinases, particularly IKKα and IKKβ, result in the nuclear translocation of NF-κB and activation of target genes. Furthermore, the selective regulation of NF-κB transcriptional activity depends on the phosphorylation of certain protein sites. Phosphorylation of NF-κB subunits may modulate transcriptional responses in a gene-specific manner [14].

Our data indicate that two distinct RelA (p65) phosphorylation events took place in rEAE: increased phosphorylation at serine 276 at the earliest stage of the disease, which was not blocked by the IKKα/β inhibitor, and increased phosphorylation at serine 536 at the later stages, accompanied by IKK kinase phosphorylation, which both were blocked by the IKK inhibitor. It should be noted that IKKα/β inhibitor not only did not decrease Ser276 phosphorylation, but doubled it compared to the level in untreated EAE mice. Therefore, there was an opposite relationship between Ser276 phosphorylation and Ser536 phosphorylation in this multiple sclerosis model.

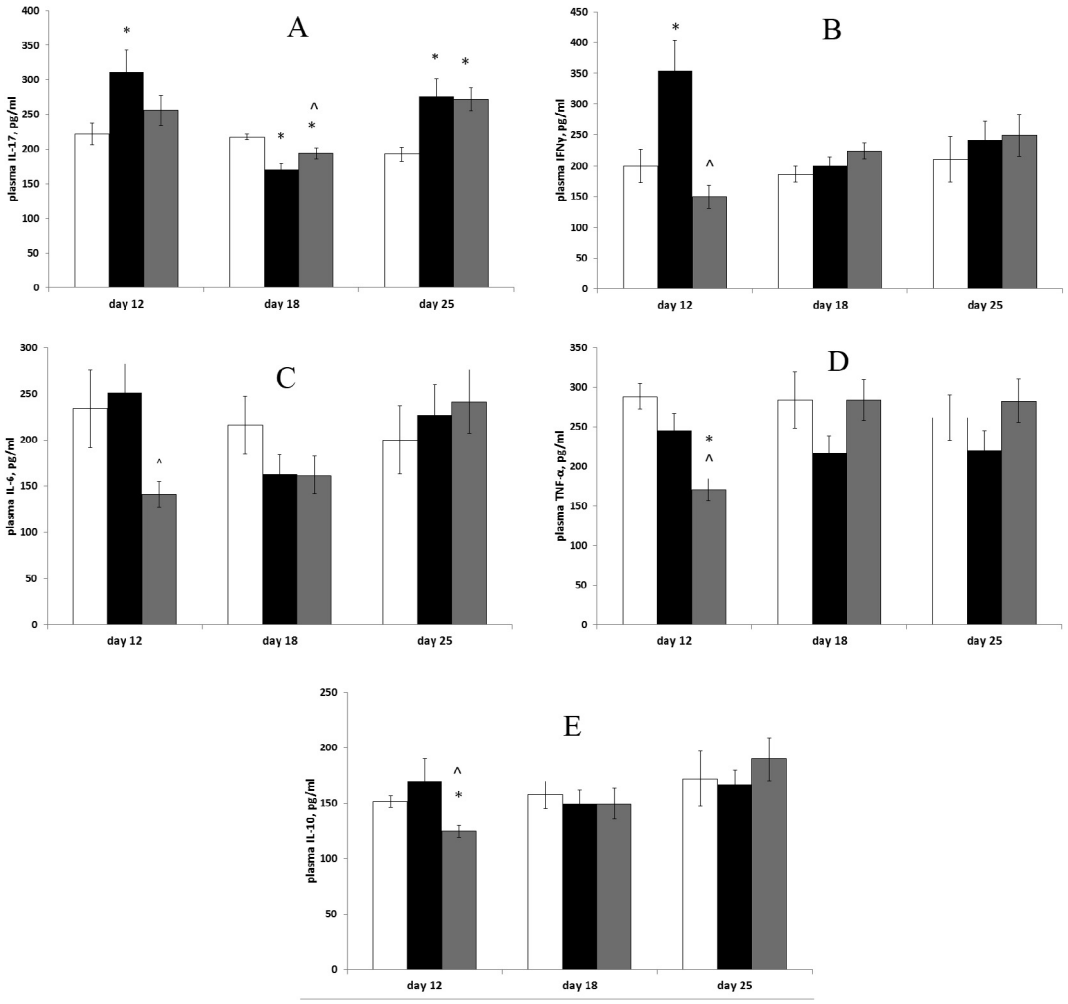

Fig. 2. Plasma cytokine concentrations throughout the observation period. Animal groups: untreated experimental autoimmune encephalomyelitis (EAE) mice, EAE mice treated with Inhibitor XII (inh), and age-matched controls. A, IL-17A; B, interferon-γ; C, IL-6; D, TNF-α; E, IL-10. Measurements were made by ELISA at different times after the immunization (day 0). The values are expressed as an average of the means from five independent experiments (ng/mL) ± standard error of the mean. Within each separate measurement, the samples were concurrently measured in quadruplicate. The averaged values from five experiments were analyzed to determine the significance of the differences between groups (n = 5). Vehicle-treated age-matched animals served as control. *Significantly different from control group, $p < 0.05$. ^Significantly different from EAE group, $p < 0.05$.

Ser276 of p65 is generally thought to be phosphorylated by PKA [14]. S276 phosphorylation triggers a conformational change in p65 that promotes its interaction with CBP/p300 and thereby increases p65 transcriptional activity [20]. Another node for the regulation of the cytosolic PKA-NF-κB complex exists through the cross-talk of the NF-κB and glucocorticoid receptor (GR) pathways [34]. The repression of GR activity by p65 requires PKA phosphorylation of Ser276, whereas GR-mediated inhibition of NF-κB activity is also PKA-dependent. Thus, the cross-repression of NF-κB and GR activity is regulated by PKA-associated signaling. Ser276 can also be phosphorylated by mitogen- and stress-activated kinase (MSK) 1/2. This MSK1/2-dependent phosphorylation is activated by a number of stimuli, including IL-1 and TNFα [35], and it has been shown to promote SCF and IL-8 expression during inflammation [36].

IKK was identified as a kinase for the p65 Ser536 site over 15 years ago in studies that revealed a dual role for the IKK complex in the activation and regulation of NF-κB [37]. During T cell activation, IKK phosphorylates Ser536 within the cytoplasmic IκB:p65 complex, an event that requires prior phosphorylation of IκB by IKK [27]. However, it has also been suggested that p65 phosphorylated on serine 536 is not associated with or regulated by IκB: it has a distinct set of target genes and may represent a non-canonical NF-κB pathway that is independent of IκB regulation [28].

In the present study, we observed that RelA/p65 protein phosphorylation at serine 276 coincided in time with Th1 cytokine production, early Th17 cytokine production, and an increase in Hsp72 expression level, whereas phosphorylation of RelA/p65 protein at serine 536 coincided with the activation of IKK, SAPK/JNK and p53 pathways, as well as later-stage Th17 cytokine production. Therefore, p65 phosphorylation may reflect different stages of the autoimmune response. It is possible that the initial stage of the process is mediated through Th1 cells and reflects the break of immunological tolerance during EAE induction. At the intracellular level, it may be related to the activation of PKA that phosphorylates p65 at Ser276, leading to the repression of glucocorticoid system activity. Moreover, PKA pathway activation has been shown to increase IL-17 production in vitro [38], which is consistent with our results. Furthermore, the observed rise in the level of Hsp72 may be also mediated by PKA, because PKA is known to phosphorylate heat shock factor 1 that migrates into the nucleus and activates Hsp70 protein expression [39]. Apparently, this stage was not blocked by IKKα/β inhibition as EAE developed despite the administration of Inhibitor XII.

The secondary stage may reflect the maintenance of the autoimmune process. The key players during this stage were likely Th17 cells. This stage was characterized by the activation of IKK that phosphorylated p65 at S536, leading to the transactivation of NF-κB and

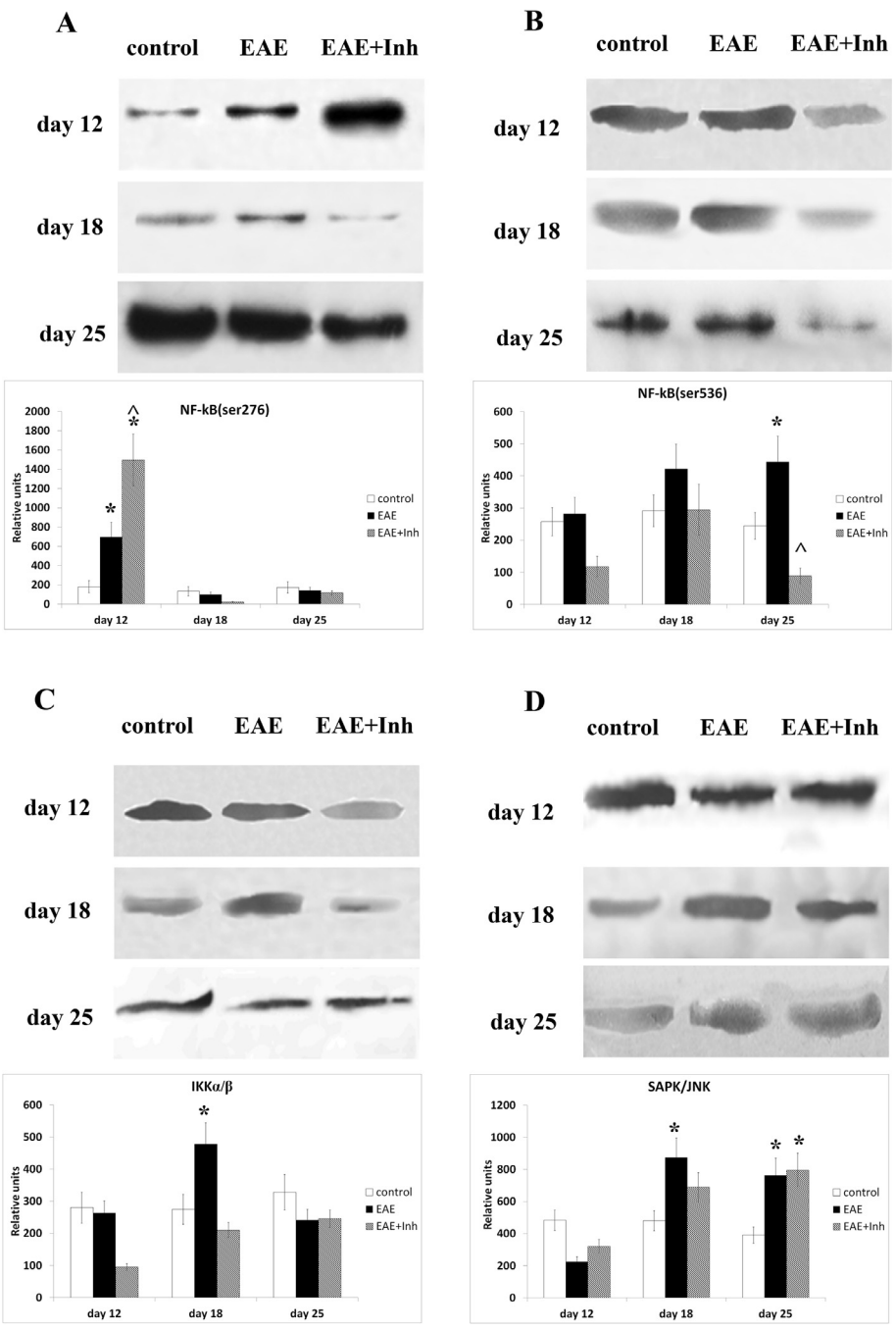

Fig. 3. Phosphorylation levels of p65/RelA at serine 276 (A) and serine 536 (B), IκB kinase (C), and SAPK/JNK in splenic lymphocytes throughout the course of disease. Animal groups: untreated mice (experimental autoimmune encephalomyelitis, EAE), EAE mice treated with inhibitor XII (EAE + inh), and age-matched controls. Protein levels were measured in spleen lymphocytes. Equal amounts of protein were analyzed at different times after the immunization (day 0) by western blot analysis using the corresponding antibodies. Histograms show the amounts of protein (± standard error of the mean) relative to those of the non-phosphorylated p65/RelA, IKK, or SAPK/JNK control and internal GAPDH control (not shown) determined by protein blot densitometry using QAPA software from three independent experiments. *Significantly different from control group, $p < 0.05$. ^Significantly different from EAE group, $p < 0.05$.

production of inflammation mediators. Probably, the inflammatory response in rEAE was balanced by some anti-inflammatory mechanisms, as evidenced by the cyclic character of symptoms and variations of IL-17 level in plasma. The administration of the IKK inhibitor shifted the balance and led to the recovery in many cases. However, if the recovery cannot be achieved, the dysregulation may follow, accompanied by SAPK/JNK pathway activation. The activation of the SAPK/JNK pathway may lead to the phosphorylation of p53, a pro-apoptotic protein known to be a SAPK/JNK pathway substrate [40].

5. Conclusion

The autoimmune process is a complex multistage process that affects many players and levels of regulation. The role of NF-κB pathway regulation through different phosphorylation sites during autoimmune pathologies is a poorly studied area. However, our data highlighted some biochemical changes that may be exploited in therapy, although this area needs further investigations.

Acknowledgements

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Conflicts of interests

None.

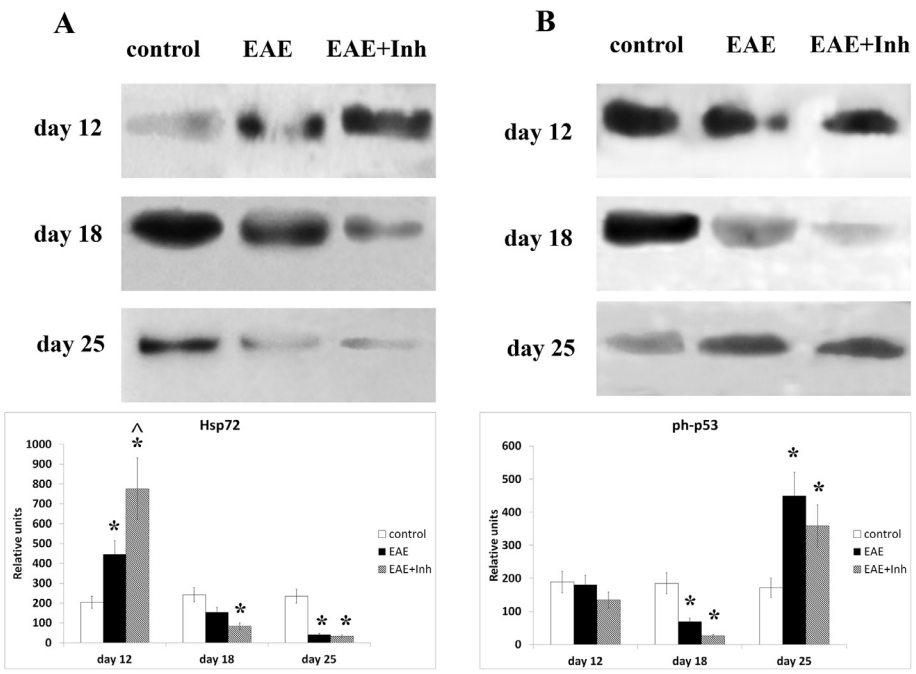

Fig. 4. Expression of Hsp72 (A) and phosphorylation of p53 (B) in splenic lymphocytes throughout the course of disease. Animal groups: untreated mice (experimental autoimmune encephalomyelitis, EAE), EAE mice treated with inhibitor XII (EAE + inh), and age-matched controls. Protein levels were measured in spleen lymphocytes. Equal amounts of protein were analyzed at different times after the immunization (day 0) by western blot analysis using corresponding antibodies. Histograms show the amount of protein (± SEM) relative to that of total SAPK/JNK, or p53, and internal GAPDH control (not shown) and are the results of protein blot densitometric measurements by QAPA software from three independent experiments. *Significantly different from control, $p < 0.05$. ^Significantly different from EAE, $p < 0.05$.

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