Transcriptional regulation of the Th17 immune response by IKKα

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Th17 cells are a subset of T cells that play crucial roles in the pathogenesis of many inflammatory diseases. We report here the identification of IKKα (inhibitor of NF-κB kinase-α) as a key transcriptional regulator of the Th17 lineage. T cells expressing a nonactivatable form of IKKα were significantly compromised in their ability to produce IL-17 and to initiate neural inflammation. IKKα is present in the nuclei of resting CD4+ T cells. Upon Th17 differentiation, IKKα selectively associated with the Il17a locus, and promoted its histone H3 phosphorylation and transcriptional activation in a NF-κB–independent manner. These findings indicate that nuclear IKKα maintains the Th17 phenotype by activating the Il17a gene.

For many years, CD4+ T helper (Th) cells have been classified into two major types, Th1 and Th2 cells (Romagnani, 1997). Th1 cells express IFN-γ and control cellular immunity, whereas Th2 cells produce IL-4, IL-5, and IL-13 and regulate humoral immunity. Recently, a new helper T cell subset, Th17 (also known as Thi), which produces IL-17A, IL-17F, IL-21, and IL-22, but not IFN-γ or IL-4, has been defined (Cua et al., 2003; Langrish et al., 2005). Transcriptionally, RORγT and RORα are considered to be master regulators of Th17 differentiation, as T-bet and GATA3 are to Th1 and Th2 cells, respectively (Ivanov et al., 2006; Dong, 2008; Yang et al., 2008). Moreover, similar to Th1 and Th2 cells in which the Ifng and Il4 loci are selectively activated, respectively, differentiated Th17 cells exhibit unique epigenetic modifications of the Il17a locus (Akinzhanov et al., 2007). However, the nuclear factors that are responsible for Il17a locus activation are not well understood.

The inhibitor of nuclear factor-κB kinase-α (IKKα) is a member of the IKK family, which regulates multiple biological processes through either NF-κB–dependent or –independent mechanisms (Häcker and Karin, 2006). IKKα can phosphorylate NF-κB2 (p100), leading to the generation of p52, which dimerizes with RelB, to activate target genes involved in lymphoid organ development (Senfleben et al., 2001). However, it has recently been recognized that IKKα can also regulate gene expression in an NF-κB–independent manner. Unlike IKKβ, IKKα contains a nuclear localization sequence. It was suggested that in the nucleus, IKKα phosphorylates histone H3 at serine (Ser) 10...
position, a prerequisite event for subsequent histone acetylation and gene transcription (Anest et al., 2003; Yamamoto et al., 2003). However, H3 Ser10 phosphorylation may simply serve as an indicator of an active “open” chromatin structure, and its dependence on IKKα may indicate that IKKα is required for establishment of the active chromatin state. More recently, IKKα kinase activity was shown to be required in the nucleus for repression of certain genes (Sil et al., 2004; Luo et al., 2007). Additionally, IKKα can also regulate epidermal keratinocyte differentiation through a kinase-independent mechanism (Hu et al., 2001). The NF-κB-independent functions of IKKα remain to be fully established.

To determine whether IKKα is required for T cell differentiation and T cell–mediated autoimmune encephalomyelitis, we studied myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) in Ikκα<sup>AA</sup> knock-in mice (Senftleben et al., 2001). The knock-in allele specifies expression of a variant IKKα protein, in which the activating phosphorylation sites, Ser176 and Ser180, are replaced by two alanines (AA), thereby abolishing the activation of its kinase activity (Bonizzi et al., 2004; Lawrence et al., 2005). We found that Ikκα<sup>AA</sup> mutant mice were refractory to EAE, and Ikκα<sup>AA</sup> CD4<sup>+</sup> T cells were defective in their Th17 cell differentiation. We then discovered that IKKα controls Th17 lineage commitment by maintaining the activation state of the I<sup>H<sub>17a</sub></sup> locus in an NF-κB–independent manner.

**RESULTS**

Autoimmune encephalomyelitis is markedly reduced in Ikκα<sup>AA</sup> knock-in mice

To determine the roles of IKKα in T cell–mediated inflammation, we immunized WT and Ikκα<sup>AA</sup> C57BL/6 mice with MOG peptide, and monitored daily for clinical signs of EAE (Fig. 1 a). Although all WT mice developed EAE, only 77.8% of Ikκα<sup>AA</sup> mice developed the disease. The disease severity was also significantly reduced in the Ikκα<sup>AA</sup> group (maximal clinical score, 1.2 ± 0.8) as compared with the WT group (maximal clinical score, 3.3 ± 0.4; P < 0.05). The day of disease onset was increased from 11.9 ± 0.9 d in the WT group to 15 ± 1.8 d in the Ikκα<sup>AA</sup> group (P < 0.01; Fig. 1 a). Consistent with these clinical findings, histological examination of spinal cord sections revealed significant differences in the degree of inflammation between the two groups. In the WT group, multiple inflammatory foci were observed, with extensive leukocyte infiltration in the white matter (Fig. 1 d, top). In contrast, leukocyte infiltration in Ikκα<sup>AA</sup> spinal cords was much less pronounced (Fig. 1 d, bottom). Thus, IKKα kinase activity contributes to the development of EAE.

Loss of IKKα kinase activity in CD4<sup>+</sup> T cells is responsible for the abrogated EAE development in Ikκα<sup>AA</sup> mice

Ikκα<sup>AA</sup> mice have severe defects in secondary lymphoid organogenesis and develop only rudiments of certain lymph nodes (Senftleben et al., 2001). This defect is caused by the loss of IKKα activity in nonhematopoietic stromal cells, other than hematopoietic cells (Senftleben et al., 2001; Bonizzi et al., 2004). To separate the effect of IKKα on lymphoid organogenesis from its effect on EAE, we studied the disease in irradiated WT C57BL/6 mice that

![Figure 1. IKKα expressed by T cells is required for the development of autoimmune encephalomyelitis.](image)
had received bone marrow from either WT or Ikka^AA^ mice (Fig. 1 b). In the chimeric mice, 80–90% of leukocytes were derived from donor bone marrow as determined by flow cytometry (unpublished data). Importantly, mice that received Ikka^AA^ bone marrow developed significantly less severe EAE than those reconstituted with WT cells (maximal disease score, 3.0 ± 0.5 vs. 4.3 ± 0.5; P < 0.01). Disease onset was also delayed from 11.0 ± 0 in the WT to 14.3 ± 0.9 d (P < 0.01) in the Ikka^AA^ group (Fig. 1 b). Therefore, loss of IKKK kinase activity in hematopoietic cells alone is sufficient to compromise the development of EAE.

Although MOG-induced EAE is a T cell–dependent disease, other hematopoietic cell types also contribute to the development of the disease. To directly test the T cell–specific function of IKKK in EAE, we studied disease development in Rag1^−/−^ mice that had received WT or Ikka^AA^ CD4^+^ T cells (Fig. 1 c). Rag1^−/−^ mice receiving WT CD4^+^ T cells started to develop clinical signs of EAE at 25.3 ± 0.6 d after MOG immunization, and reached a maximal disease score of 3.7 ± 0.3. Remarkably, although Rag1^−/−^ mice received Ikka^AA^ CD4^+^ T cells developed EAE with only a slight delay relative to mice reconstituted with WT cells (28.3 ± 0.5 d; P < 0.01), the severity of the disease was significantly reduced (maximal disease score: 0.7 ± 0.3; P < 0.01). All mice in this experiment developed EAE, but no mice died of the disease. Splenic T cell numbers of RAG1 knockout recipient mice were determined 22 d after the disease onset. No significant differences were observed between mice that received WT or IKKK^AA^ cells. Collectively, these results establish that IKKK kinase activity in CD4^+^ T cells is essential for the development of EAE.

It is to be noted that in addition to IKKK^AA^ expressed by T cells, IKKK^AA^ expressed by non–T cells (hematopoietic and non-hematopoietic cells) may also play a role in EAE. However, this issue is difficult to address because of the following: (a) IKKK^AA^ expressed by nonhematopoietic cells may indirectly affect EAE by controlling lymphoid organogenesis, in addition to its potential direct effects in the CNS; (b) the difference in EAE between bone marrow chimeras (Fig. 1 b) and their parental mice (Fig. 1 a) is small. Thus, in this study we focused on the roles of IKKK in T cells.

To measure the anti-MOG response of transferred T cells, Rag1^−/−^ mice were sacrificed 22 d after disease onset, and their splenocytes were cultured in the presence of MOG peptide (Fig. 2). Total splenic T cell and splenocyte numbers were not significantly different between mice received WT and Ikka^AA^ cells. Splenocytes isolated from mice receiving WT T cells proliferated vigorously in response to MOG peptide and produced high levels of IL-2, IL-4, IL-17A, and IFN-γ. Strikingly, splenocytes from mice reconstituted with Ikka^AA^ T cells exhibited significantly reduced proliferation (P < 0.05) and cytokine production (P < 0.01; Fig. 2). Similar reduction in MOG-induced responses was observed in Ikka^AA^ splenocytes isolated from the mice tested in Fig. 1 (a and b; and not depicted). To determine...
whether IKKα mutation affects the survival of CD4+ T cells, we performed flow cytometry analysis of blood samples collected from mice reconstituted with T cells before they were immunized for EAE. We observed no significant differences in the number and frequency of T cells between WT and Ikkα44 groups. Additionally, WT and Ikkα44 T cells, when cultured in the presence of plate-bound anti-CD3 and soluble anti-CD28, did not have significant differences in survival as determined by flow cytometry after staining the cells with Annexin V. In contrast, Ikkα-deficient T cells had a significantly increased rate of death under the same culture condition (unpublished data).

IKKα kinase activity is required for Th17 responses
The markedly reduced anti-MOG response of Ikkα44 CD4+ T cells indicates that the IKKα kinase activity may be required for T cell activation and/or differentiation. To test this theory, we isolated CD4+ T cells from naive WT and Ikkα44 mice and measured their responses to anti-CD3 and anti-CD28 stimulation (Fig. 3). We found that Ikkα44 mutant CD4+ T cells proliferated to the same extent as WT CD4+ T cells after anti-CD3 or anti-CD3 plus anti-CD28 stimulation (Fig. 3a). Furthermore, upon stimulation with anti-CD3 and anti-CD28, Ikkα44 CD4+ T cells produced normal levels of IL-2, IL-4, and IFN-γ as compared with WT cells; however, when stimulated with anti-CD3 alone, they produced moderately less IL-4 and IFN-γ (Fig. 3). In contrast, Ikkα44 CD4+ T cells produced significantly less IL-17A than WT cells upon stimulation with anti-CD3 or anti-CD3 plus anti-CD28 (Fig. 3e). IL-17A, but not IL-2, mRNA expression in Ikkα44 CD4+ T cells was also significantly reduced (Fig. S1). The viability of T cells in the two groups was not significantly different, as determined by trypan blue and/or Annexin V staining. Similar results were obtained when unfractionated WT and Ikkα44 splenocytes were stimulated with anti-CD3 and anti-CD28 (unpublished data). These results are in contrast to the global defect of the Ikkα44 CD4+ T cells to MOG restimulation as shown in Fig. 2. Because T cells tested in those experiments were isolated from mice with different degrees of EAE, the effect of the disease on T cell responsiveness could not be excluded. Ikkα44 mice did not show general defects in T cell activation, and similar percentages of CD4+CD44+ T cells (15.5 ± 1.3% vs. 16.3 ± 1.7%) and CD4+CD62Llow T cells (22.6 ± 2.1% vs. 24.6 ± 1.6%) were detected in spleens of naive WT and Ikkα44 mice, respectively.

These results indicate that IKKα may selectively regulate Th17 cell differentiation. To test this possibility, we compared the ability of WT and Ikkα44 CD4+ T cells to differentiate into Th17 cells in vitro. Differentiated Th17 cells were identified by flow cytometry after intracellular staining of IL-17A. When cultured under Th0 conditions, very few Th17 cells were spontaneously generated, but ~14% of WT or Ikkα44 CD4+ T cells produced IFN-γ (Fig. 4a). In contrast, when cultured under Th17-inducing conditions, 9.9 ± 0.6% of WT CD4+ T cells produced IL-17; but only 5.6 ± 0.8% of Ikkα44 T cells were IL-17+ (P < 0.01; Fig. 4b). Therefore, IKKα kinase activity is required for the development of the Th17 response.

IKKα specifically regulates Il17a gene expression
To determine whether IKKα regulates Th17 response at the transcriptional level, we examined the expression of a panel of Th17-related genes by quantitative real-time PCR. Il17a expression in Ikkα44 CD4+ T cells was significantly reduced as compared with WT cells when cultured under either Th0 or
IKKα selectively binds to the Il17a promoter

IKKα has been previously reported to be present in the nuclei of several nonhematopoietic cell types, such as keratinocytes, squamous epithelial cells, and fibroblasts (Anest et al., 2003; Yamamoto et al., 2003; Fernández-Majada et al., 2007). In the nucleus, IKKα can regulate target gene expression through a variety of mechanisms, some of which correlate with promoter-associated histone H3 phosphorylation (Anest et al., 2003; Yamamoto et al., 2003). Interestingly, we found that IKKα was not only present in the cytoplasm, but also constitutively expressed at high levels in the nuclei of resting CD4+ T cells (Fig. 5a). The level of nuclear IKKα was ~30% of the cytoplasmic IKKα, and was not regulated by anti-CD3 or anti-CD28 stimulation. To test whether IKKα regulates Il17a expression by a direct association with its promoter, we performed chromatin immunoprecipitation (ChIP) with anti-IKKα (Fig. 5). IKKα did not bind to the Il17a promoter in resting WT CD4+ T cells, but in cells that had been cultured in the Th17 differentiation medium for 1–2 d, IKKα was readily detected on the Il17a promoter, coinciding with the appearance of the IL-17A mRNA (Fig. 5, b and c). The Il17a promoter was selectively targeted by IKKα, as IKKα was not detected on promoters of Il21, Il17f, and Il2 (Fig. 5c).

In WT T cells, IKKα binding to the Il17a promoter correlated with its histone H3 phosphorylation (Fig. 5 d). In contrast, in Ikka-/- T cells, mutant IKKα also bound to the Il17a promoter under Th17 differentiation conditions, but histone H3 phosphorylation did not occur (Fig. 5 d). On the other hand, histone H3 on Il17a promoter also underwent phosphorylation at serine10 position, 1 and 2 d after cells were cultured in Th17 differentiation medium, but this event was

Th17 condition (Fig. 4 c and Fig. S2). In contrast, Il17f (Fig. 4 d) and Il23R (not depicted) expressions were not affected by the loss of IKKα kinase activity. Il21 expression was only marginally reduced in Ikka-/- CD4+ T cells (Fig. 4 e). Additionally, IL-17A heterogenous nuclear RNA (hnRNA) could be readily detected in activated WT CD4+ T cells; in contrast, a much reduced signal was detected in Ikka-/- T cells under the same condition (Fig. S3). The Th17 differentiation signals elicited by TGF-β and IL-6 eventually converge onto the induction of two Th17 lineage-specific transcription factors, RORα and RORγT (Ivanov et al., 2006; Dong, 2008). However, the expression of Rora and Rorgt mRNAs and RORγ and RORγT proteins was not affected by the IKKα mutation (Fig. 4, f and g, and Fig. S4). These data indicate that the effect of IKKα on Th17 lineage is Il17a specific and independent of RORα and RORγT.

Figure 4. Defective IL-17 production and Th17 differentiation of Ikka-/- CD4+ T cells.

CD4+ T cells from WT (n = 4) and Ikka-/- (n = 4) mice were cultured under either neutral (Th0) or Th17-inducing condition as indicated. (a) 72 h later, IL-17– and/or IFN-γ–producing cells were measured by flow cytometry after intracellular staining of cytokines. (b) Quantification of data shown in a. (c–g) 24 h after the initiation of the culture, total RNA was isolated and mRNA levels of the Th17 lineage genes were assessed by real-time RT-PCR. The lower expression level of each gene was set to 1. The experiments were repeated at least three times with similar results. *, P < 0.05; **, P < 0.001.
IKKα–independent (Fig. 5 d). As expected, IKKα mutation selectively affected PolII binding to Il17a, but not to Il21 and Il22 (Fig. S5). These results indicate that IKKα kinase activity is selectively required for driving the Il17a locus into an active state marked by H3 serine 10 phosphorylation.

**IKKα regulates Th17 response independent from NF-κB**

In addition to directly acting on the Il17a promoter, IKKα may also regulate Th17 response independent from NF-κB–dependent mechanisms. To test this possibility, we first examined IκBα degradation in WT and Ikkα−/− T cells. In WT CD4+ T cells, upon anti-CD3 and anti-CD28 stimulation, IκBα was quickly degraded followed by a recovery caused by resynthesis of IκBα (Fig. 6 a, left). The early degradation of IκBα in Ikkα−/− CD4+ T cells was not affected, but IκBα did not return to the prestimulation level at later time points. Similar results were obtained when cells were stimulated with PMA plus ionomycin (unpublished data). To test whether IKKα regulates the IκBα promoter as previously reported (Anest et al., 2003; Yamamoto et al., 2003), we performed ChIP–PCR analyses using CD4+ T cells stimulated with anti-CD3 and anti-CD28. We found that IKKα was recruited to IκBα promoter in both WT and Ikkα−/− CD4+ T cells after stimulation (Fig. 6 c). However, H3 Ser10 phosphorylation on the Iκbα promoter was detected only in WT, but not in mutant, T cells. These results indicate that IKKα kinase activity is required for IκBα resynthesis, but not for its stimulation-induced degradation in T cells.

Next, we measured nuclear translocation of NF-κB subunits in T cells stimulated with anti-CD3 and anti-CD28. RelA, RelB, c-Rel, and p50 all migrated into the nucleus of WT CD4+ T cells upon activation (Fig. 6 a). As expected (Bonizzi et al., 2004), the IKKα mutation did not affect the nuclear translocation of RelA, c-Rel, or p50, but completely blocked nuclear localization of RelB (Fig. 6 a), indicating that IKKα selectively controls RelB activation. The lack of an effect of IKKα mutation on nuclear RelA activity was also confirmed by RelA ELISA (Fig. 5 e). As the key kinase of the noncanonical (alternative) NF-κB pathway, IKKα mediates the processing of p100 and, consequently, the generation of the RelB-p52 heterodimer (Bonizzi et al., 2004). Defective RelB nuclear translocation in Ikkα−/− CD4+ T cells may be caused by impaired IKKα-regulated p100 processing. We therefore examined p100 processing and p52 nuclear translocation in anti-CD3– and anti-CD28–activated T cells (Fig. 6 b). In WT T cells, p100 and p52 were up-regulated in both the cytosolic and nuclear fractions upon stimulation. In Ikkα−/− T cells, however, p100 was also up-regulated in the cytosol and nucleus, but p52 expression was reduced in the cytosol and was undetectable in the nucleus after anti-CD3 and anti-CD28 stimulation. These data confirm that IKKα kinase activity is important for activation–induced p100 processing and is required for p52 and RelB nuclear translocation in T cells. Similar results were obtained when WT and Ikkα−/− T cells were stimulated with PMA and ionomycin (unpublished data).

These results raise the question of whether IKKα regulates Th17 cell differentiation through p52–RelB heterodimers. To test this, we examined whether RelB was required for Th cell differentiation by culturing WT and Relb−−/− CD4+ T cells under Th1- or Th17-inducing conditions (Fig. 6 d).
data). Expression of two Th17 lineage-specific transcription factors, RORα and RORγT, was not changed in Ikkα-/- CD4+ T cells, indicating that the defect may be attributed to another mechanism. This theory is supported by the observation that IKKα is recruited to the Il17a promoter during Th17 differentiation and a specific defect in IL-17A expression in Ikkα-/- CD4+ T cells. Furthermore, phosphorylation of histone H3 on the Il17a promoter, an activation marker that correlates with Th17 differentiation, was absent in Ikkα-/- T cells. Therefore, our study suggests a novel nuclear function for IKKα in Th17 differentiation.

In this study, we showed that RelB activation after anti-CD3 plus anti-CD28 stimulation was blocked in Ikkα-/- CD4+ T cells. Although it has been reported that RelB is required for maximal T cell activation (Corn et al., 2005), we did not observe any defect in Ikkα-/- CD4+ T cell proliferation and Th1 and Th2 cytokine production after anti-CD3 and anti-CD28 stimulation; this may be caused by additional effects of the IKKα mutation, such as reduced IkBα resynthesis, which may compensate for the loss of RelB function. However, when stimulated in vitro with MOG peptide, spleenocytes isolated from Ikkα-/- mice that had developed EAE showed significantly reduced responses (Fig. 2). There are two possible reasons for this discrepancy. First, IKKα may be required for T cell activation induced by weak, but not strong,
ligands. Second, and more likely, the decreased anti-MOG T cell response may be secondary to the effect of IKKα on EAE. The increased severity of EAE in WT mice may help to activate and expand more MOG-specific CD4+ T cells than in Ikkα−/− mice. As a consequence, there are likely more MOG-responsive T cells in the WT splenocyte culture than in the Ikkα−/− culture (Fig. 2).

It should be emphasized that IKKα-mediated IL-17 regulation may be only one of the mechanisms whereby IKKα controls EAE, as other cytokines including Th1 cytokines may also be affected by IKKα (Figs. 2 and 3). With regard to IL-17, the most significant IKKα effect appears to be on its mRNA transcription (Figs. 3 and 4). However, because IKKα does not appear to affect the IL-17 protein levels of individual Th17 cells, but increases the frequency of IL-17–producing cells, we propose that IKKα may selectively affect IL-17 mRNA expression in a subpopulation of Th17 cells, but not all Th17 cells. This could be related to the stage of Th17 cell differentiation, the phase of cell cycle, and the microenvironment surrounding the Th17 cells. This may explain why many Ikkα−/− cells do not produce any IL-17 protein, although others make normal amounts (Fig. 4).

Two subunits of the IKK complex, IKKα and IKKβ, possess kinase activities (Häcker and Karin, 2006). Despite high sequence similarities, it is now evident that the two molecules possess distinct functions (Häcker and Karin, 2006). In T cells, IKKβ mediates NF-κB activation through the canonical pathway (Schmidt-Supprian et al., 2003). Ikkα−/− CD4+ T cells show significantly less IkBα degradation with severely delayed kinetics (unpublished data). In contrast, IKKα was not required for the activation of the canonical NF-κB pathway in CD4+ T cells. Instead, IKKα is required for the activation of the noncanonical NF-κB pathway through p100 processing (Fig. 6 b). In the cytosol, p100 can still be processed, albeit at a markedly reduced rate in Ikkα−/− cells. However, p52 nuclear translocation was completely abolished in these cells.

Gene expression is regulated by epigenetic mechanisms involving histone modifications. Dong et al. showed that similar to loci specific to Th1 and Th2 cells, Th17 loci encoding IL-17A and IL-17F were regulated by chromatin remodeling events (Akimzhanov et al., 2007). Transcriptionally permissive histone modifications, such as histone H3 acetylation and Lys-4 tri-methylation, were observed at the Il17a promoter in Th17 cells (Akimzhanov et al., 2007). We showed that during Th17 differentiation, histone H3 on both Il17a and Il17f promoters was phosphorylated at Ser10, a known prerequisite for histone H3 acetylation. However, IKKα was found to be selectively required for histone H3 phosphorylation at the Il17a promoter, but not Il17f promoter.

Recently, several groups have reported nuclear expression of IKK subunits (Anest et al., 2003; Yamamoto et al., 2003; Fernández-Majada et al., 2007; Lubin and Sweat, 2007). Unlike IKKβ and IKKγ (NEMO), which are exclusively cytoplasmic, IKKα contains a nuclear localization sequence. Furthermore, IKKα was found to be recruited to NF-κB–dependent (e.g., Il8, Il6, and IkBa) and NF-κB–independent (e.g., c-fos) gene promoters, where its presence correlated with H3 phosphorylation, and thus chromatin activation. However, nuclear IKKα was also found to be recruited to genes (e.g., Maspin) that undergo active repression (Luo et al., 2007). Although IKKα was proposed to directly phosphorylate histone H3 at Ser10 (Anest et al., 2003; Yamamoto et al., 2003), its association with gene repression and silencing suggest that IKKα–induced enhancement of H3 phosphorylation can also be mediated by indirect mechanisms. For example, IKKα may be responsible for the recruitment of identified H3 kinases to the promoters it activates. In addition, nuclear IKKα has also been shown to mediate RelA/p65 turnover in macrophages through its phosphorylation of Ser536 (Lawrence et al., 2005). This effect seems to be cell-type specific because we found that IKKα was not required for RelA/p65 phosphorylation on Ser536 in CD4+ T cells (unpublished data). This may partially explain the different roles of IKKα in T cells as compared with macrophages. Thus, the highly specific effect of IKKα on Th17 lineage and the resistance of Ikkα−/− mice to MOG–induced EAE implicate IKKα as a potential therapeutic target for Th17–mediated autoimmune disorders.

MATERIALS AND METHODS

Mice and cell transfer. Ikkα−/− mutant C57BL/6 mice were previously described (Senftleben et al., 2001). To generate bone marrow chimeric mice, C57BL/6 recipient mice were irradiated with 2 doses of 500 rads each. They were then intravenously injected with 105 bone marrow cells collected from either WT or Ikkα−/− mice. 8 wk after bone marrow reconstitution, mice were immunized for the induction of EAE as described below. For T cell transfer, 105 CD4+ T cells isolated from WT or Ikkα−/− mice were injected into Rag1−/− C57BL/6 mice through the tail vein. 24 h after the transfer, mice were immunized with MOG for EAE induction. All procedures were preapproved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

Cell isolation, cell culture, and reagents. Naïve CD4+CD25−CD44− CD62L+ T cells were isolated by FACs, whereas total CD4+ T cells were isolated by MACS. The purity of the naïve T cells isolated by FACs was >99%, whereas that of T cells isolated by MACS was >95%. Cells were cultured in complete DME containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 30 µM β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM Hepes, and 1% nonessential amino acids. Anti-IKKα, anti-RelA, anti-RelB, and anti-c-Rel were purchased from Santa Cruz Biotechnology, Inc. Anti–β-actin was purchased from Sigma-Aldrich. Anti-CD3 and anti-CD28 were purchased from ebioscience. Anti-IkBα, anti-pRelA536, anti-p110/52, and anti-pSer10H3 were purchased from Cell Signaling Technology. MOG 38–50 peptide was synthesized by Invitrogen. Pertussis toxin was purchased from List Biological Laboratories, Inc. CFA was purchased from DIFCO laboratories.

EAE induction and evaluation. EAE was induced as previously described (Hillard et al., 2002). In brief, mice first received a subcutaneous immunization with 300 µg MOG38–50 peptide emulsified in CFA and an intravenous injection of 200 ng pertussis toxin. A second injection of 200 ng pertussis toxin was given 48 h later. Mice were examined daily for clinical signs of EAE and scored as follows: 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb plus forelimb paralysis; 5, moribund or dead.

Th cell differentiation. CD4+ T cells were isolated using autoMACS automatic cell sorters (Miltenyi Biotec). Cells were cultured with 50 U/ml IL-2,
2 µg/ml anti-CD28, and 2 µg/ml anti-CD3 under either Th0- (no more addition of cytokines or antibodies), Th1- (10 ng/ml IL-12 and 20 ng/ml anti- IL-4), or Th17- (0.4 µg/ml anti-IL-4, 10 µg/ml anti-IFN-γ, 20 ng/ml IL-6, and 5 µg/ml TG-Fβ) inducing conditions. 5 d later, cells were washed and restimulated with anti-CD3 and anti-CD28 for 12 h, stained with anti-IL-17A and anti-IFN-γ, and examined by flow cytometry.

Real-time PCR. Total RNA was extracted using TRizol reagent (Invitrogen). cDNA was synthesized using reverse transcription II (Invitrogen). Real-time quantitative PCR was performed using the SYBR Green master mix (Applied Biosystems). Data were normalized to β-actin mRNA. Primers used are as follows (forward and reverse): IL-17A, 5'-ACCTCACCAGGGGACGAAATG-3' and 5'-CCCTTACAGGGGTGATCC-3'; IL-17F, 5'-GAGGATACGACTGAGTTGAGTCGAC-3' and 5'-GAGGTCTAGTGCGTCTTCCTC-3'; IL-21, 5'-CTCCATGAGCCTCTGCGGCCC-3' and 5'-ATCGTACTTCTCCATGCAATCCC-3'; IκBα, 5'-TCTCCCTGCGCTTCGCAC-3' and 5'-TCACAGACCTGTCTGAGGA-3'; RORγt, 5'-CATACAGAAGGCGTTCATTG-3' and 5'-CCGGCTGAGGGCCTTAC-3'; RORγt, 5'-CCGGCTGAGGGCCTTAC-3' and 5'-GAGCAGGATGATGTCATTGC-3'; IL-12, 5'-GAGAAGCTGGTGAGCGAGCTG-3' and 5'-GAGGGAGGATGGGGTGGG-3'; RORγt, 5'-TCAGTGCTACAATCTTCTTCAGAGGACA-3' and 5'-TCCTTGTGGAGCAGCA-3'; β-actin, 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CITCTTTGTAGTCAGCCGACATTT-3'.

ChIP PCR. ChIP was performed using a Chromatin Immunoprecipitation kit (Millipore) according to the manufacturer's instructions. In brief, cells were first treated with formaldehyde and sonicated to break up the chromatin. Sonicated chromatin preparations were immunoprecipitated with specific antibodies. DNA was then eluted after extensive washing. PCR was performed using the SYBR Green master mix (Applied Biosystems) per manufacturer's instructions. Total cell lysate was prepared using a Western blot. Western blot. Nuclear protein extract was prepared using a Nuclear Extract kit (Active Motif Inc.) per manufacturer's instructions. cDNA was synthesized using reverse transcription II (Invitrogen). Real-time PCR. Quantitative ELISA was performed using paired mAbs specific for corresponding cytokines or antibodies, Th1- (10 ng/ml IL-12 and 20 ng/ml anti-IL-4) or Th17- (0.4 µg/ml anti-IL-4, 10 µg/ml anti-IFN-γ, 20 ng/ml IL-6, and 5 µg/ml TG-Fβ) inducing conditions. 5 d later, cells were washed and restimulated with anti-CD3 and anti-CD28 for 12 h, stained with anti-IL-17A and anti-IFN-γ, and examined by flow cytometry.

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