# NIK signaling in dendritic cells but not in T cells is required for the development of effector T cells and cell-mediated immune responses

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**The canonical NF-κB pathway is a driving force for virtually all aspects of inflammation. Conversely, the role of the noncanonical NF-κB pathway and its central mediator NF-κB–inducing kinase (NIK) remains poorly defined. NIK has been proposed to be involved in the formation of $T_H17$ cells, and its absence in $T_H$ cells renders them incapable of inducing autoimmune responses, suggesting a T cell–intrinsic role for NIK. Upon systematic analysis of NIK function in cell-mediated immunity, we found that NIK signaling is dispensable within CD4+ T cells but played a pivotal role in dendritic cells (DCs). We discovered that NIK signaling is required in DCs to deliver co-stimulatory signals to CD4+ T cells and that DC-restricted expression of NIK is sufficient to restore $T_H1$ and $T_H17$ responses as well as cell-mediated immunity in *NIK*$^{-/-}$ mice. When CD4+ T cells developed in the absence of NIK-sufficient DCs, they were rendered anergic. Reintroduction of NIK into DCs allowed developing *NIK*$^{-/-}$ CD4+ T cells to become functional effector populations and restored the development of autoimmune disease. Therefore, our data suggest that a population of thymic DCs requires NIK to shape the formation of most αβ CD4+ T effector lineages during early development.**

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Abbreviations used: BMC, BM chimeric mouse; cDC, conventional DC; CNS, central nervous system; DTx, diphtheria toxin; EAE, experimental autoimmune encephalomyelitis; mTEC, medullary thymic epithelial cell; NIK, NF-κB–inducing kinase; $nT_{reg}$ cell, natural regulatory T cell; pDC, plasmacytoid DC; qRT-PCR, quantitative RT-PCR; SLT, secondary lymphoid tissue; SP, single positive.

Noncanonical NF-κB signaling is a prerequisite for the anlage of secondary lymphoid tissues (SLTs). Mice carrying lesions in elements of this pathway are often alymphoplastic (absence of lymph nodes) and lack the specific lymphoid organization in spleen and thymus (Weih and Caamaño, 2003). The notion that cell-mediated immunity commences exclusively in SLTs provides a tangible explanation for the immunodeficiency of alymphoplastic mice. Because of their inability to generate germinal centers, alymphoplastic mice such as lymphotoxin-β receptor–deficient (*LTβR*$^{-/-}$), *LTα*$^{-/-}$, *NIK*$^{aly/aly}$, and *NIK*$^{-/-}$ animals are all defective in immunoglobulin class-switch (Miyawaki et al., 1994; Banks et al., 1995; Shinkura et al., 1996; Fütterer et al., 1998). However, T cell responses and cell-mediated immunity are severely reduced in *NIK*$^{aly/aly}$ mice when compared with other alymphoplastic mice (Greter et al., 2009).

NF-κB–inducing kinase (NIK) is a key mediator of the noncanonical NF-κB pathway (Sun and Ley, 2008). It transduces signals from distinct members of the TNFR family and induces via phosphorylation of IκB-specific kinase α (IKK-α) the cleavage of p100-RelB to p52-RelB, which then translocates as heterodimer into the nucleus (Senftleben et al., 2001; Xiao et al., 2004). The activity of NIK is tightly regulated on several levels, generally using the TNFR-associated factors 2/3 (TRAF2/3), cytosolic inhibitor of apoptosis 1 (cIAP1), and cIAP2 (Varfolomeev et al., 2007; Vince et al., 2007), which prevent basal activation of this pathway. The signal-induced activation of the noncanonical pathway results in the degradation of TRAF2 and TRAF3 and thus in the

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stabilization of NIK protein (Liao et al., 2004). *NIK*$^{aly/aly}$ mice contain a point mutation that is located in the C-terminal region of NIK and is responsible for the physical interaction with the upstream TRAFs and IKK-α (Shinkura et al., 1999). Thus, the levels of nuclear p52 in several tissues and cell types of *NIK*$^{aly/aly}$ mice are virtually ablated (Xiao et al., 2001b).

There is evidence that noncanonical NF-κB signaling within hematopoietic cells is involved in several human diseases such as lymphoid cancers, including EBV-positive Hodgkin's lymphoma and HTLV-1–transformed T cell lymphoma (Xiao et al., 2001a; Atkinson et al., 2003; Eliopoulos et al., 2003). Also, mutations in NIK have been correlated with the development of multiple myeloma (Annunziata et al., 2007). Thus, NIK poses an attractive pharmacological target for the treatment of a variety of diseases (Staudt, 2010), and it is thus important that its role and function within the immune system are resolved.

For many years, it has been believed that the noncanonical NF-κB pathway is preferably activated by ligands either important for the lymphoid organogenesis (through LTβR) or in B cell responses (through CD40 and BAFF-R; Youssef and Steinman, 2006). However, it has become increasingly evident that the noncanonical NF-κB pathway can be triggered by many different ligands such as RANK, LIGHT, TWEAK, CD70, and CD28 (Darnay et al., 1999; Yin et al., 2001; Ramakrishnan et al., 2004; Sánchez-Valdepeñas et al., 2006; Nadiminty et al., 2007; Bhattacharyya et al., 2010; Maruyama et al., 2010; Sanz et al., 2010). Furthermore, it was reported that NIK can also signal into the classical NF-κB pathway (Ramakrishnan et al., 2004; Zarnegar et al., 2008; Staudt, 2010; Sasaki et al., 2011). The vast variety of triggers suggests that noncanonical NF-κB signaling is not exclusively active in the development of SLTs but also plays a role in B and T cell responses as well as in the function of APCs. NIK-deficient T cells have been shown to be defective in secretion of IL-2 and GM-CSF (Sánchez-Valdepeñas et al., 2006). They are further limited in their proliferative capacity as well as $T_H17$ differentiation and fail to become pathogenic in experimental autoimmune encephalomyelitis (EAE), graft versus host disease, and in models of transplantation (Yamada et al., 2000; Matsumoto et al., 2002; Ishimaru et al., 2006; Sánchez-Valdepeñas et al., 2006, 2010; Greter et al., 2009; Jin et al., 2009).

Apart from the involvement in T cell function, alternative NF-κB signaling has been controversially discussed in the induction of central tolerance by regulating the development and function of Aire$^+$ medullary thymic epithelial cells (mTECs; Chin et al., 2003; Kajiura et al., 2004; Venanzi et al., 2007; Akiyama et al., 2008). The impaired mTEC function in *NIK*$^{aly/aly}$ or *RelB*-deficient mice provides a potential explanation for the reported autoimmune phenotypes. However, the reason for disturbed cell-mediated immunity in *NIK*$^{aly/aly}$ animals remains poorly understood.

Even though the immunodeficiency in *NIK*$^{aly/aly}$ mice was thought to be caused by their alymphoplasia, we have previously reported that the defect in cell-mediated immunity in *NIK*$^{aly/aly}$ mice is not connected to their lack of SLTs but that NIK activity is critical for cellular immune function (Greter et al., 2009). Cell-mediated immunity could be induced in the total absence of SLTs when splenectomized *NIK*$^{aly/aly}$ mice were reconstituted with a WT hematopoietic system. However, cell-mediated immunity could not be induced in mice featuring normal SLTs but carrying the *NIK*$^{aly/aly}$ lesion only in hematopoietic cells (Greter et al., 2009). Although a critical function for NIK has been suggested specifically in T cells by several studies (Yamada et al., 2000; Matsumoto et al., 2002; Ishimaru et al., 2006; Sánchez-Valdepeñas et al., 2006; Jin et al., 2009), the data presented in this study suggest that the loss of T cell function in *NIK*$^{aly/aly}$ mutants was not T cell intrinsic, but rather resulted from a defect in hematopoietic accessory leukocytes, namely DCs.

We show in this study that NIK-lesioned T cells were indeed capable of secreting effector cytokines and acquiring full effector functions, depending on the thymic environment in which they developed. Instead of a T cell–intrinsic lesion, loss of NIK in thymic DCs imprinted a long-lasting halt in T cell effector function. However, if NIK-deficient T cells matured within a thymic environment that hosts NIK, T cells gained proper effector functions regardless of their NIK deficiency. Thus, we propose that thymic DCs, which so far have only been implemented in negative selection (Gallegos and Bevan, 2004) and the induction of natural regulatory T cells ($nT_{reg}$ cells; Proietto et al., 2009), are capable of imprinting subsequent T cell effector function onto developing αβ thymocytes. This ability of thymic DCs is strictly dependent on NIK.

## RESULTS

### Loss of NIK function results in reduced T cell proliferation, differentiation, and production of effector cytokines

We have recently reported that *NIK*$^{aly/aly}$ mice are resistant to the induction of EAE because of the loss of function of NIK within the hematopoietic compartment and more specifically because of a defect in T cell priming, independent of the lack of SLTs (Greter et al., 2009). Furthermore, *NIK*$^{aly/aly}$ T cells have been reported to be defective in proliferation and secretion of IL-17, IL-2, and GM-CSF (Matsumoto et al., 2002; Sánchez-Valdepeñas et al., 2006; Jin et al., 2009). In line with these previous studies, we observed that in vitro, polyclonally activated *NIK*$^{aly/aly}$ CD4$^+$ T cells produced lower amounts of effector cytokines (IL-2, IFN-γ, and IL-17) than heterozygous *NIK*$^{aly/+}$ controls, whereas the production of IL-4 was unaffected (Fig. 1 A). This suggests a T cell intrinsic impairment of polarization conferred by the ablation of NIK signaling. To further investigate the requirement of NIK for antigen-specific T cell activation, we crossed *NIK*$^{aly/aly}$ mice with TCR transgenic 2d2 mice, in which the TCR recognizes the immunodominant epitope of the myelin oligodendrocyte glycoprotein ($MOG_{35–55}$). As expected, *NIK*$^{aly/aly}$-2d2 T cells also failed to secrete effector cytokines upon encountering their cognate antigen (Fig. 1 B). To exclude the possibility that the observed defects were caused by the developmental

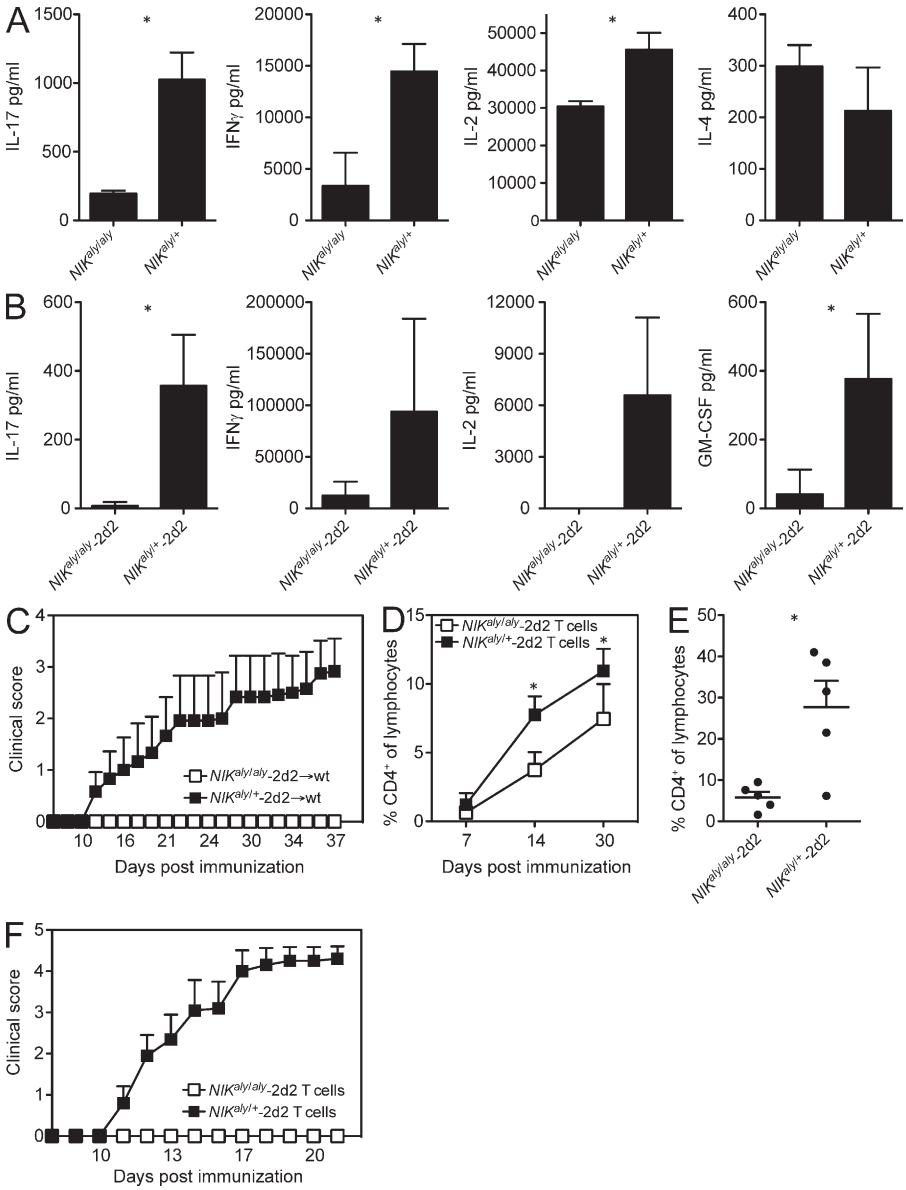


**Figure 1. *NIK*$^{aly/aly}$ mice are resistant to EAE because of the lack of NIK signaling in immune cells.** (A) CD4$^+$ T cells of *NIK*$^{aly/aly}$ or *NIK*$^{aly/+}$ mice were stimulated in vitro with plate-bound α-CD3/α-CD28 for 48 h. Cytokine secretion was analyzed by ELISA. (B) Splenocytes from *NIK*$^{aly/aly}$-2d2 or *NIK*$^{aly/+}$-2d2 mice were stimulated in vitro with $MOG_{35–55}$ and α-CD28 for 48 h. Cytokine secretion was analyzed by ELISA. (C) *NIK*$^{aly/aly}$-2d2 → WT or *NIK*$^{aly/+}$-2d2 → WT BMCs were immunized with $MOG_{35–55}$/CFA and monitored daily for clinical signs of EAE ($n = 6$). (D–F) CD4$^+$ T cells from *NIK*$^{aly/aly}$-2d2 → WT or *NIK*$^{aly/+}$-2d2 → WT BMCs were transferred into *Rag1*$^{-/-}$ mice. Homeostatic expansion was observed by analyzing the percentage of CD4$^+$ T cells within total lymphocytes in the peripheral blood (D). 30 d after adoptive CD4$^+$ T cell transfer, *Rag1*$^{-/-}$ mice were immunized with $MOG_{35–55}$/CFA and observed for antigen-driven expansion by FACS analysis of blood at day 7 after immunization (E) and clinical signs of EAE ($n = 6$; F). Each graph shows one representative of three independent experiments. (A, B, D, and E) Error bars indicate SD. *, $P \leq 0.05$. (C and F) Error bars indicate SEM.

malformations of SLTs in *NIK*$^{aly/aly}$ mice, we generated BM chimeric mice (BMCs) in which WT mice were reconstituted with hematopoietic stem cells from either *NIK*$^{aly/aly}$-2d2 or *NIK*$^{aly/+}$-2d2 mice. We found that even if most of the CD4$^+$ T cells carry the cognate antigen-specific TCR, *NIK*$^{aly/aly}$-2d2 → WT BMCs retained their EAE resistance upon $MOG_{35–55}$/CFA immunization (Fig. 1 C), emphasizing the critical role of NIK signaling for the development of T cell–mediated autoimmune responses.

Because T cells from *NIK*$^{aly/aly}$-2d2 → WT BMCs failed to acquire pathogenic properties, we addressed their behavior in antigen-independent homeostatic expansion in lymphopenic *Rag1*$^{-/-}$ mice. After adoptive transfer of CD4$^+$ T cells from *NIK*$^{aly/aly}$-2d2 → WT BMCs into *Rag1*$^{-/-}$ mice, we observed a reduction in homeostatic expansion when compared with CD4$^+$ T cells from *NIK*$^{aly/+}$-2d2 → WT BMCs (Fig. 1 D). Upon immunization of those mice with $MOG_{35–55}$/CFA, *NIK*$^{aly/aly}$-2d2 T cells further failed to respond to their cognate antigen, whereas control *NIK*$^{aly/+}$-2d2 T cells strongly expanded (Fig. 1 E). In addition, *Rag1*$^{-/-}$ mice reconstituted with T cells from *NIK*$^{aly/aly}$-2d2 → WT BMCs remained completely resistant to EAE, suggesting that *NIK*$^{aly/aly}$-2d2 T cells cannot be primed by NIK-sufficient accessory cells (Fig. 1 F). Collectively, the data support the notion that NIK deficiency indeed leads to a T cell–intrinsic lesion.

### Loss of NIK results in a primary APC defect

Our data and a previous study (Jin et al., 2009) support a T cell–intrinsic role of NIK in cell-mediated immunity. However, we further aimed to identify the role of NIK in the accessory cell compartment. BMCs were generated by transferring a 4:1 mixture of *Rag1*$^{-/-}$ and *NIK*$^{aly/aly}$-2d2 BM into *Rag1*$^{-/-}$ mice. In those mice, *NIK*$^{aly/aly}$-2d2 T cell progenitors are developing within an NIK-sufficient accessory cell environment. Surprisingly, *Rag1*$^{-/-}$ + *NIK*$^{aly/aly}$-2d2 → *Rag1*$^{-/-}$ BMCs developed EAE comparable with *NIK*$^{aly/+}$-2d2 → *Rag1*$^{-/-}$ BMCs (Fig. 2 A). This finding demonstrates that NIK plays a vital role in hematopoietic accessory cells rather than in T cells to develop autoimmune inflammation. Complementing this result, polyclonally in vitro activated CD4$^+$ T cells

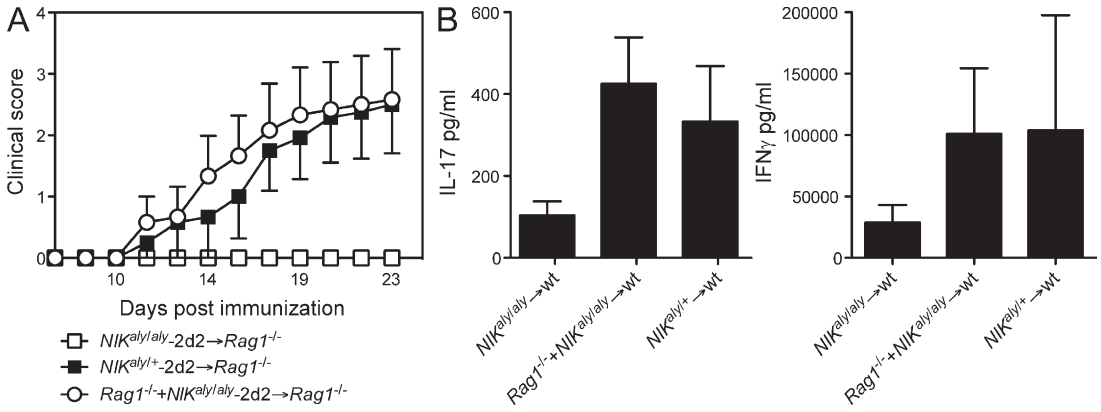


**Figure 2. Loss of NIK signaling in accessory cells determines the fate of T cells early in development.** (A) Lethally irradiated *Rag1*$^{-/-}$ mice were reconstituted with BM of *NIK*$^{aly/aly}$-2d2, *NIK*$^{aly/+}$-2d2, or a 4:1 mixture of *Rag1*$^{-/-}$ and *NIK*$^{aly/aly}$-2d2 mice. 6 wk after reconstitution, BMCs were immunized with $MOG_{35-55}$/CFA and observed for clinical signs of EAE ($n = 6$). Error bars indicate SEM. (B) Lethally irradiated WT mice were reconstituted with BM of *NIK*$^{aly/aly}$, *NIK*$^{aly/+}$, or a 4:1 mixture of *Rag1*$^{-/-}$ and *NIK*$^{aly/aly}$ mice. 6 wk after reconstitution, $CD4^+$ T cells were isolated and stimulated with plate-bound α-CD3/α-CD28. Supernatants were analyzed by ELISA. Each graph shows one representative of three independent experiments. Error bars indicate SD.

from *Rag1*$^{-/-}$ + *NIK*$^{aly/aly}$ → WT BMCs were rescued in their ability to secrete IL-17 and IFN-γ (Fig. 2 B), suggesting that the production of effector cytokines by T cells requires intact NIK signaling in the accessory cell compartment but not the T cell compartment.

### DC maturation and co-stimulation are dependent on NIK signaling

The most prominent accessory cells involved in the induction of cell-mediated immunity and antigen presentation are DCs. It has been reported that in vitro *NIK*$^{aly/aly}$ DCs have defects in the expression of CD80, CD86, and MHCII as well as in antigen presentation and their ability to drive T cell expansion (Garceau et al., 2000; Tamura et al., 2006; Lind et al., 2008). Also the ability of T cells to secrete effector cytokines is largely dependent on the capacity of APCs to provide T cell instructive cytokines. In particular, the cytokines IL-12, IL-23, and IL-6 have a major impact on the polarization of effector T cells. We investigated the ability of splenic *NIK*$^{aly/aly}$ DCs to secrete these factors after stimulation with anti-CD40, thereby mimicking T cell–APC interactions. Interestingly, activated *NIK*$^{aly/aly}$ DCs secreted significantly lower levels of the proinflammatory cytokine subunit IL-12/IL-23p40 (Fig. 3, A and B; and Fig. S1, A and B). We could further observe a strong reduction of the transcripts for *IL-12p35* and *IL-23p19* (Fig. S1 C) and protein levels of IL-6 (Fig. 3 C). These data suggest that NIK signaling in DCs upon DC–T cell interaction via CD40 is critical for the capacity of DCs to secrete T cell-instructive cytokines.

### *NIK*$^{aly/aly}$ DCs are restrained in T cell priming and fail to induce EAE

To verify the reduced priming capacity of *NIK*$^{aly/aly}$ DCs in vivo, we established a model based on diphtheria toxin (DTx)–mediated cell ablation. We created mice with an immune compartment containing both NIK-deficient and -sufficient T cells, whereas the vast majority of DCs carry the mutated *NIK*$^{aly/aly}$ protein. To do so, a 1:1 mixture of *CD11cDTR* and *NIK*$^{aly/aly}$ BM was transferred into irradiated WT recipients (Fig. 4 A). Upon injection of DTx, DCs of *CD11cDTR* origin (*NIK*$^{+/+}$) were depleted, whereas mutant *NIK*$^{aly/aly}$ DCs were retained. The efficiency of DC ablation in *CD11cDTR*

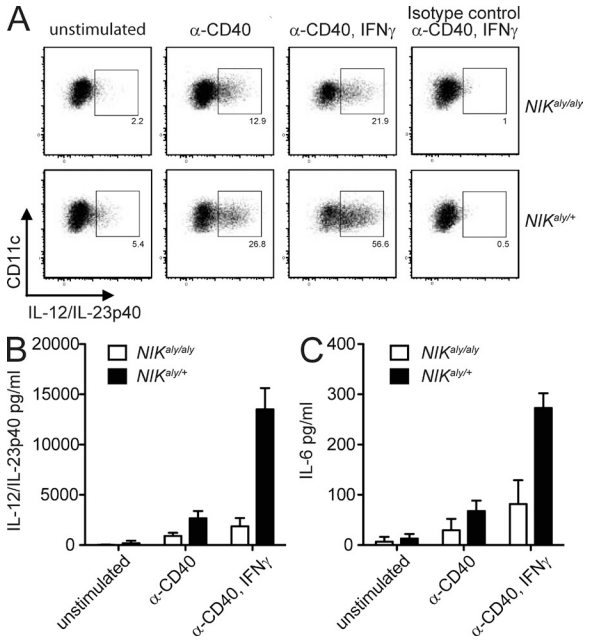


**Figure 3. *NIK*$^{aly/aly}$ DCs are restrained in secretion of proinflammatory cytokines.** (A–C) Splenic *NIK*$^{aly/aly}$ or control DCs were stimulated in vitro with α-CD40 and IFN-γ for 24 h. GolgiPlug was added for the last 4 h of culture. Intracellular anti–IL-12/IL-23p40 FACS staining was performed together with cell surface staining for CD11c (A). Supernatants were analyzed by ELISA for IL-12/IL-23p40 (B) and IL-6 (C). Each graph shows one representative of three independent experiments. (B and C) Error bars indicate SD.

mice was >90% (Fig. 4 B). We observed a significant delay in disease onset (Fig. 4 C) with decreased central nervous system (CNS) infiltration of T cells (Fig. 4 D), and particularly IL-17–producing T cells (Fig. 4 E), in DTx-treated *CD11cDTR* + *NIK*$^{aly/aly}$ → WT BMCs compared with DTx-treated control *CD11cDTR* + *NIK*$^{aly/+}$ → WT BMCs. This finding strongly supports our previous in vitro (Fig. 3) and in vivo (Fig. 2 A) data and demonstrates that NIK signaling in DCs is critical for their ability to instruct T cell polarization and effector function.

### Restoration of NIK signaling in DCs is sufficient to generate pathogenic T cells

To ascertain a primary role of NIK signaling in the DC compartment, we generated mice in which NIK expression is restricted to DCs. *R26Stop*$^{FL}$*NIK*$^{WT}$ mice express *NIK*$^{WT}$ preceded by a loxP-flanked neo$^{R}$-Stop cassette and followed by an Frt-flanked *IRES-eGFP* within the ubiquitously active *ROSA26* locus (Sasaki et al., 2008). Upon crossing to *CD11c-cre* mice, the neo$^{R}$-Stop is excised, and *NIK*$^{WT}$ will be expressed in CD11c$^{+}$ cells, which are mainly DCs (these mice are hereafter called DC$^{NIK}$). Upon further breeding those animals onto the *NIK*$^{-/-}$ background, we generated mice that express NIK$^{WT}$ in DCs, whereas other cells and tissues lack the ability to express NIK. To verify the cell type–specific targeting of the NIK$^{WT}$ transgene, we analyzed GFP expression in different immune compartments, which confirmed the transgene expression in DCs (Fig. S2 A).

To manipulate the expression of NIK only within the hematopoietic compartment and to provide a normal lymphoid organ structure, we again generated BMCs by transferring BM of either DC$^{NIK}$-*NIK*$^{-/-}$ or control DC$^{NIK}$-*NIK*$^{+/-}$ and *NIK*$^{-/-}$ mice into lethally irradiated WT mice. 6 wk after reconstitution, these BMCs were immunized with MOG$_{35–55}$/CFA and observed for the development of clinical disease. Strikingly, DC$^{NIK}$-*NIK*$^{-/-}$ → WT BMCs were fully susceptible to EAE, even though most T cells are *NIK*$^{-/-}$ (Fig. 5 A). Furthermore, the secretion of proinflammatory cytokines IL-12/IL-23p40 and IL-6 in DCs of DC$^{NIK}$-*NIK*$^{-/-}$ → WT BMCs was largely restored (Fig. 5 B). Also, the restoration of NIK$^{WT}$ in DCs rescued the secretion of T effector cytokines IL-17, IFN-γ, and GM-CSF (Fig. 5 C) after antigen restimulation. These findings demonstrate that NIK signaling in DCs is sufficient to generate autoaggressive CD4$^{+}$ T cells, even if these T cells themselves do not express functional NIK.

In addition to verifying the transgene expression in DCs, we thoroughly analyzed other leukocyte populations for inadvertent ectopic expression. Macrophages were found to be negative, and although microglia can express transient levels of CD11c upon activation, they can be excluded as effectors in this model because *NIK*$^{-/-}$ → WT BMCs have NIK-sufficient microglia but remained EAE resistant (Fig. 5 A). In DC$^{NIK}$-*NIK*$^{-/-}$ → WT

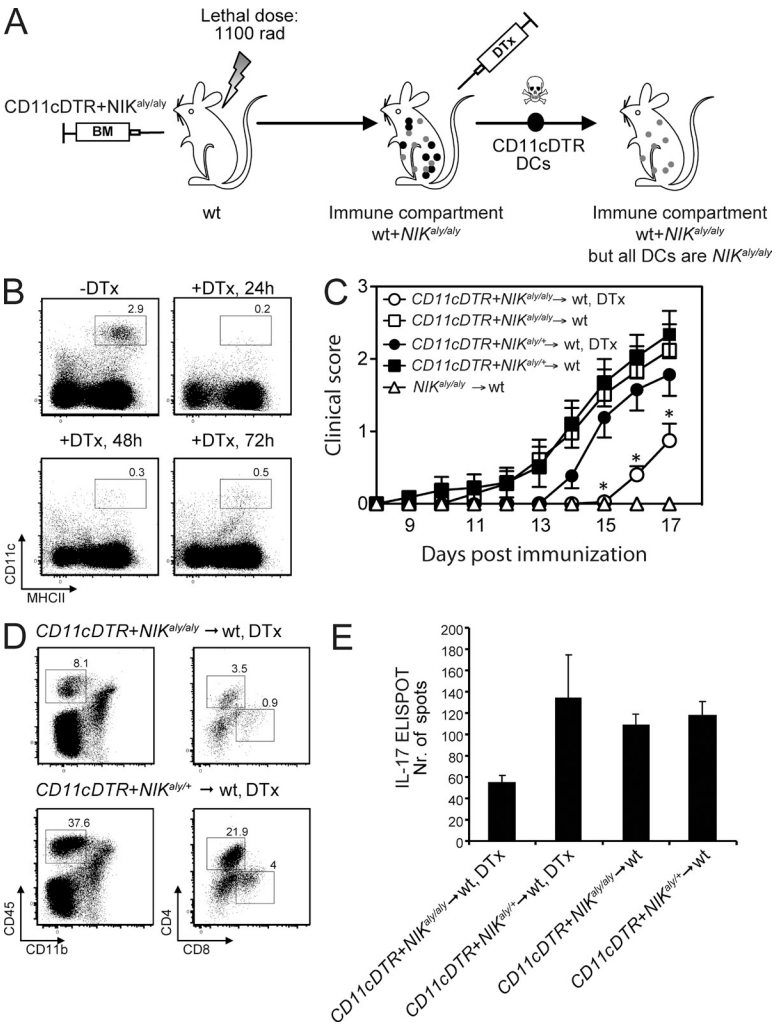


**Figure 4. Absence of NIK signaling in DCs significantly delays EAE.** (A) Lethally irradiated WT mice were reconstituted with a 1:1 mixture of *CD11cDTR* and *NIK*$^{aly/aly}$ or *CD11cDTR* and *NIK*$^{aly/+}$ BM. Upon i.p. injection of DTx, *CD11cDTR* (*NIK*$^{+/+}$) DCs are depleted, whereas *NIK*$^{aly/aly}$ or *NIK*$^{aly/+}$ DCs remain. All other immune cells are still present as *NIK*$^{+/+}$. (B) Efficiency of DC depletion in DTx-treated *CD11cDTR* mice was analyzed by flow cytometry. (C) *CD11cDTR* + *NIK*$^{aly/aly}$ → WT and *CD11cDTR* + *NIK*$^{aly/+}$ → WT BMCs were immunized with MOG$_{35–55}$/CFA and treated with DTx every second day. Mice were observed for clinical signs of EAE. Three individual experiments were pooled ($n \geq 15$/group). Error bars indicate SEM. *, $P \leq 0.05$. (D) FACS analysis of CNS-infiltrating lymphocytes in MOG$_{35–55}$/CFA-immunized DTx-treated *CD11cDTR* + *NIK*$^{aly/aly}$ → WT and *CD11cDTR* + *NIK*$^{aly/+}$ → WT BMCs at peak disease (17 d after immunization). Several brains and spinal cords of each experimental group were pooled. (E) Splenocytes were isolated from MOG$_{35–55}$/CFA-immunized DTx-treated/untreated *CD11cDTR* + *NIK*$^{aly/aly}$ → WT and *CD11cDTR* + *NIK*$^{aly/+}$ → WT BMCs and rechallenged in vitro with 50 µg/ml MOG$_{35–55}$ peptide, followed by IL-17 ELISPOT. Triplicates of pooled splenocytes of one experiment are shown ($n \geq 5$/group). Error bars indicate SD.

BMCs, we detected a minor percentage of GFP$^+$CD4$^+$ and CD8$^+$ T cells (Fig. S2 A). To ensure that this population of NIK-expressing T cells does not contribute to the EAE susceptibility of DC$^{NIK}$-*NIK*$^{-/-}$ → WT BMCs, we further bred T$^{NIK}$-*NIK*$^{-/-}$ mice by crossing *R26Stop*$^{FL}$*NIK*$^{WT}$ mice to *CD4-cre* and *NIK*$^{-/-}$ mice. T$^{NIK}$-*NIK*$^{-/-}$ → WT BMCs were, similar to *NIK*$^{-/-}$ → WT BMCs, completely EAE resistant (Fig. S2 B). Thus, the reintroduction of NIK$^{WT}$ into the DC pool fully restored EAE susceptibility.

### Loss of NIK signaling critically impairs thymic DC function

Our data thus far clearly showed that NIK signaling in DCs is critical for T effector function. However, if the restoration of NIK signaling in DCs alone reinstates T effector function, the fact that adoptively transferred mature *NIK*$^{aly/aly}$ T cells into NIK-sufficient recipients fail to acquire effector function represents a contradiction (Fig. 1). Thus, we hypothesized that NIK signaling in thymic DCs is required to enable developing thymocytes to exit the thymus as fully functional T cells. Therefore, the thymic DC compartment of *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ mice was analyzed in detail. All three thymic DC subsets (Wu and Shortman, 2005; Proietto et al., 2008a; Li et al., 2009), namely migratory and resident conventional DCs (cDCs; CD11c$^+$CD172α$^+$ and CD172α$^-$, respectively) and plasmacytoid DCs (pDCs; CD11c$^{int}$CD45RA$^+$), were found in comparable numbers in *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ mice (Fig. 6 A). However, both cDCs and pDCs in *NIK*$^{aly/aly}$ mice expressed reduced levels of CD80 and CD86 and even more drastically MHCII (Fig. 6 B), with the strongest reduction in the resident DC subset. This indicates some degree of functional impairment of APC properties, which we further assessed by co-culturing *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ thymic DCs with 2d2 CD4$^+$ single-positive (SP) thymocytes in the presence of MOG$_{35–55}$. *NIK*$^{aly/aly}$ thymic resident DCs elicited reduced proliferation in 2d2 CD4$^+$ SP thymocytes (Fig. 6 C).

To further investigate the phenotypic properties of *NIK*$^{aly/aly}$ thymic DCs, we performed quantitative RT-PCR (qRT-PCR) analysis for various chemokine ligands and receptors involved in thymic DC function (Proietto et al., 2008a). *NIK*$^{aly/aly}$ thymic DCs revealed a strong reduction in the expression of *CCL17*, *CCL19*, and *CCL21* (Fig. 6 D), which are crucial for the migration of developing thymocytes (Ueno et al., 2004; Proietto et al., 2008a). Furthermore, the analysis of chemokine receptors revealed an overall reduction in the levels of *CCR2*, *CCR5*, *CCR6*, and *CCR7* but increased expression of *CCR9* and *TLR9* (Fig. S3). Collectively,

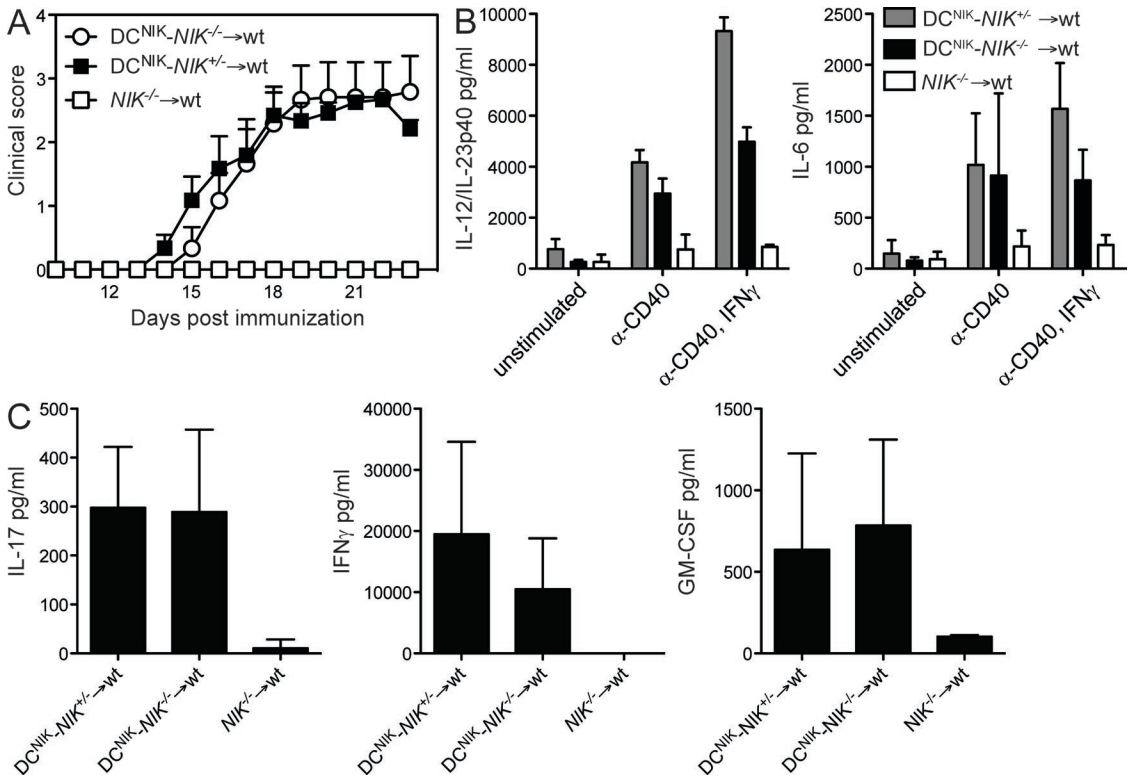


**Figure 5. Expression of NIK in DCs is sufficient to restore EAE susceptibility in *NIK*$^{-/-}$ mice.** (A) DC$^{NIK}$-*NIK*$^{-/-}$ → WT, DC$^{NIK}$-*NIK*$^{+/-}$ → WT, and *NIK*$^{-/-}$ → WT BMCs were immunized with MOG$_{35–55}$/CFA and observed for clinical signs of EAE. Shown is one representative of three independent experiments ($n$ = 6). Error bars indicate SEM. (B) Splenic DCs were isolated from DC$^{NIK}$-*NIK*$^{-/-}$ → WT and DC$^{NIK}$-*NIK*$^{+/-}$ → WT BMCs and stimulated in vitro with α-CD40 and IFN-γ for 24 h. IL-12/IL-23p40 and IL-6 secretion was measured by ELISA. (C) Splenocytes of MOG$_{35–55}$/CFA-immunized DC$^{NIK}$-*NIK*$^{-/-}$ → WT and DC$^{NIK}$-*NIK*$^{+/-}$ → WT BMCs were isolated at the peak of disease and restimulated in vitro with MOG$_{35–55}$ for 48 h. Supernatants were analyzed by ELISA. (B and C) Each graph shows one representative of three independent experiments. Error bars indicate SD.

we found that *NIK*$^{aly/aly}$ thymic DCs are phenotypically and functionally distinct from those in *NIK*$^{aly/+}$ mice, suggesting a causative link between the altered function of thymic DCs and the subsequent loss of T effector function.

### Restoration of NIK in DCs rescues Foxp3, RORγt, and Tbet expression in developing thymocytes

Thymic DCs have primarily been implicated in mediating negative selection (Gallegos and Bevan, 2004). However, there is increasing evidence that thymocyte development not only selects the T cell receptor repertoire but also imprints effector function onto thymic emigrants. In particular, NIK has been suggested to be involved in the expansion of CD25$^+$CD4$^+$ T cells (Lu et al., 2005; Tamura et al., 2006). We found a 50% reduction in FoxP3$^+$ $T_{reg}$ cells in both developing and mature T cells (Fig. 7, A and B). Given the reduced effector cytokine expression of *NIK*$^{aly/aly}$ T cells, we speculated whether the observed decrease of natural occurring $nT_{reg}$ cells in *NIK*$^{aly/aly}$ thymi is the result of altered licensing of T cells during their development. Recently, thymic T cell lineage commitment has been expanded to other lineages including $T_H17$ cells (Marks et al., 2009). Therefore, we analyzed the gene expression of lineage-specific transcription factors in CD4$^+$ SP cells of *NIK*$^{aly/aly}$ thymi (Fig. 7 C) and spleens (Fig. 7 D) and found that in addition to *Foxp3*, the expression of *RORγt* and *Tbet* was decreased, whereas *GATA3* was not affected. We further found that the percentage of IL-17– and IFN-γ–producing CD4$^+$ SP thymocytes was strongly reduced in naive *NIK*$^{aly/aly}$ mice (Fig. 7 E). Interestingly, a very recent study described that RelB, which is one of the target molecules of NIK, is essential for LTβR-dependent thymic commitment of γδ T cells toward IL-17 production (Powolny-Budnicka et al., 2011).

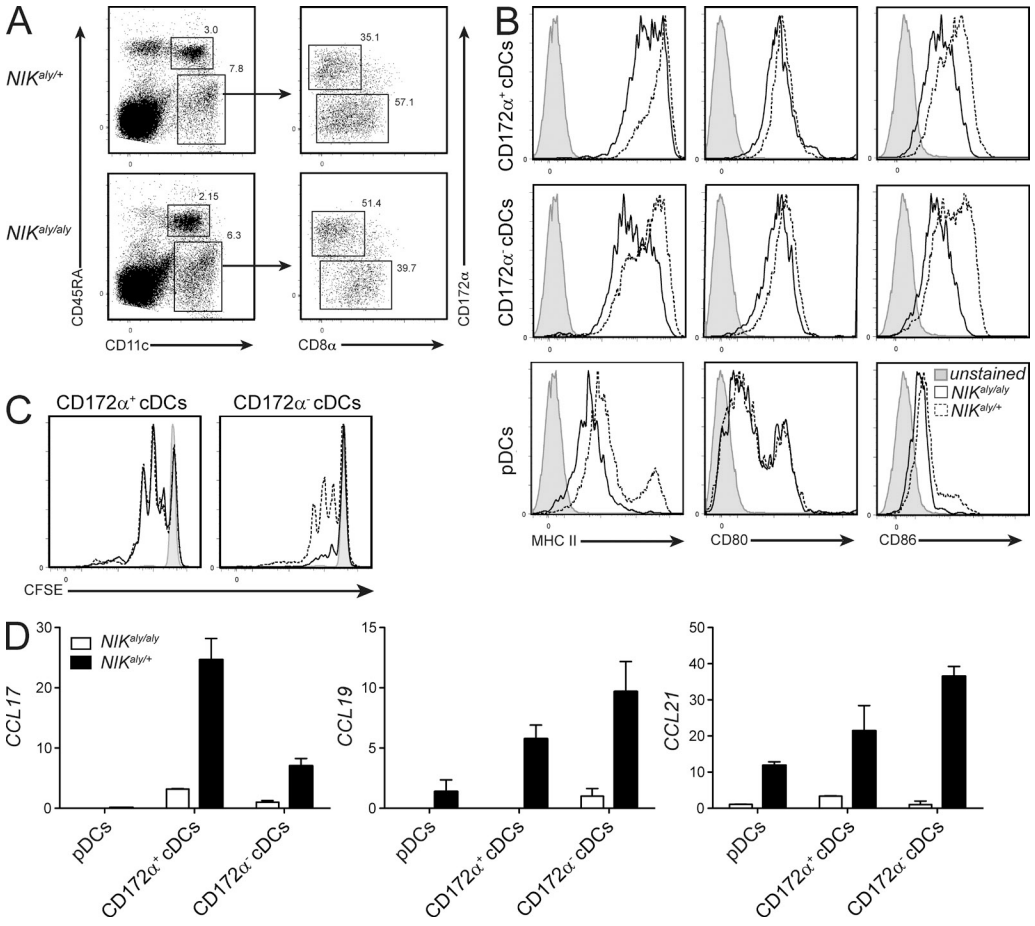


**Figure 6. *NIK*$^{aly/aly}$ thymic DCs show reduced APC capacity.** (A) Flow cytometric analysis of thymic DC subsets from *NIK*$^{aly/aly}$ and control animals. pDCs are CD11c$^{int}$CD45RA$^+$, thymic resident DCs are CD11c$^+$CD172a$^-$, and thymic migratory DCs are CD11c$^+$CD172a$^+$. Numbers in the plots indicate the percentage of the respective DC subset of the total thymic DC fraction. (B) Expression analysis of MHCII, CD80, and CD86 on thymic DC subsets. Dotted lines represent heterozygous control DCs, solid lines *NIK*$^{aly/aly}$ DCs, and gray histograms unstained controls. (C) Proliferation of CFSE-labeled 2d2 CD4$^+$ SP thymocytes after 3 d of co-culture with thymic migratory (left) and resident (right) DCs in the presence of 10 µg/ml $MOG_{35-55}$. Dotted lines represent CFSE profile after stimulation with heterozygous control DCs, solid lines show *NIK*$^{aly/aly}$ DCs, and gray histograms show unstimulated cells. (D) RNA of FACS-sorted thymic DC subsets from *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ mice was transcribed into cDNA and analyzed by qRT-PCR for expression of different chemokines. Shown is the fold change in expression level compared with CD172α$^-$ *NIK*$^{aly/aly}$ cDCs, which was set to 1. Data in all panels are representative of three independent experiments. Error bars indicate SD.

In line with this, our observations suggest that thymic commitment toward the $T_H1$ or $T_H17$ lineage might also occur for αβ T cells and that NIK signaling in thymic DCs is crucial for this process.

To verify that DC-restricted expression of $NIK^{WT}$ is capable of restoring early lineage commitment, we again generated DC-$^{NIK}$-*NIK*$^{-/-}$ → WT, DC$^{NIK}$-*NIK*$^{+/-}$ → WT, and *NIK*$^{-/-}$ → WT BMCs and analyzed the expression of transcription factors in CD4$^+$ SP thymocytes. As shown in Fig. 8 (A and B), DC-restricted $NIK^{WT}$ expression fully restored *RORγt*, *Tbet*, and also *Foxp3* expression in CD4$^+$ SP thymocytes.

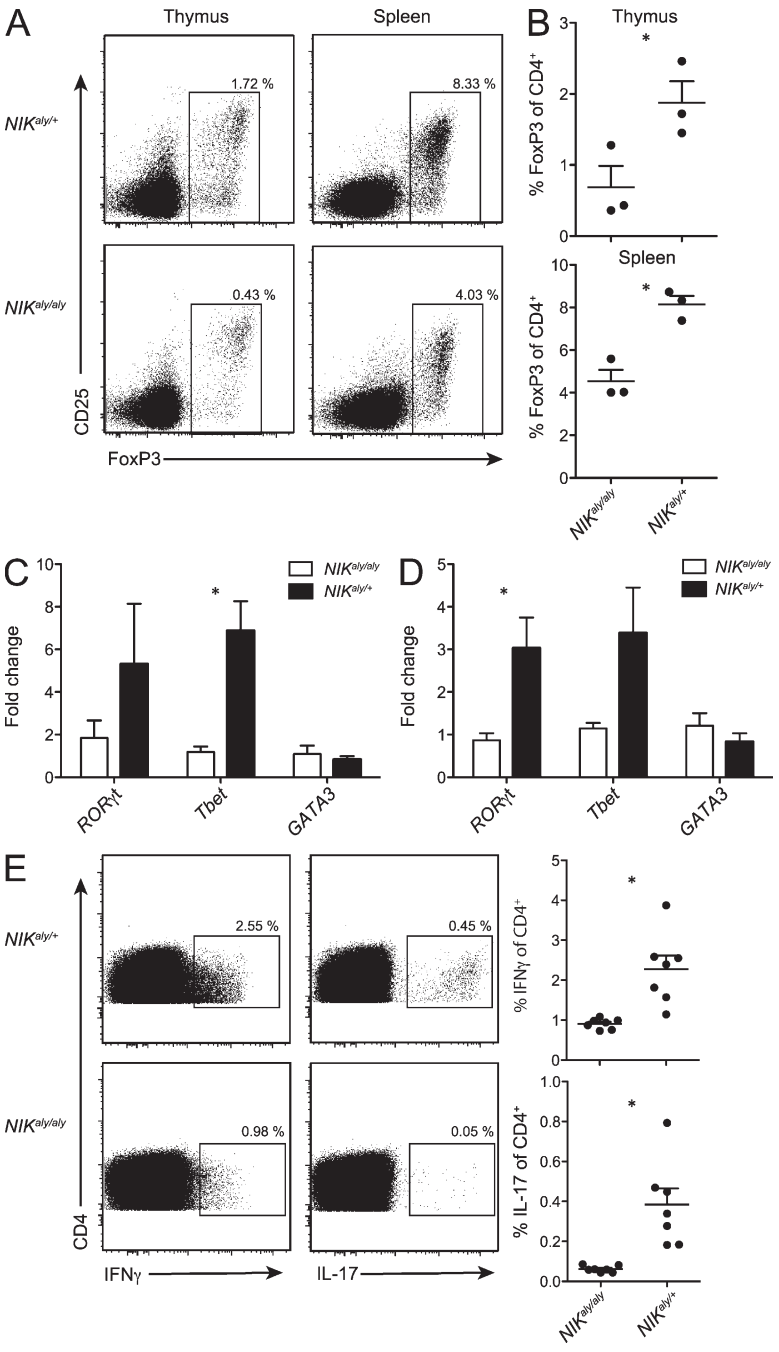


**Figure 7. *NIK*$^{aly/aly}$ mice show strongly reduced percentage of thymic committed nT$_{reg}$ cells and T$_H$17 and T$_H$1 cells.** (A and B) *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ thymocytes and splenocytes were stained intracellularly for FoxP3 expression. Plots are gated on CD4$^+$ CD8$^-$ cells (A). Shown are means of three mice each (B). (C and D) RNA of FACS-sorted SP CD4$^+$ thymic (C) and splenic (D) T cells of *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ mice was transcribed into cDNA and analyzed by qRT-PCR for expression of *RORγt*, *Tbet*, and *GATA3*. Shown is fold change of expression levels compared with one of three *NIK*$^{aly/aly}$ samples, which was set to 1. Data are representative of three independent experiments ($n = 3$). (E) *NIK*$^{aly/aly}$ and *NIK*$^{aly/+}$ CD4$^+$ SP thymocytes of naive mice were stimulated in vitro with PMA and ionomycin and stained intracellularly for the secretion of IL-17 and IFN-γ. Data are pooled from two independent experiments ($n = 7$). (B–E) Error bars indicate SD. *, $P \leq 0.05$.

## DISCUSSION

NIK is widely held as a central mediator of noncanonical NF-κB signaling and the activation of NF-κB2. Indeed, both *NF-κB2*$^{-/-}$ and *NIK*$^{aly/aly}$ mice show impaired T and B cell responses, while displaying lymphocyte infiltration into various organs similar to that of *Aire*$^{-/-}$ mice (Anderson et al., 2002; Liston et al., 2003; Kajiura et al., 2004; Zhu et al., 2006). However, the autoimmune phenotype in *NIK*$^{aly/aly}$ and *NF-κB2*$^{-/-}$ mice seems to originate from the stromal compartment, as transplantation of *NIK*$^{aly/aly}$ or *NF-κB2*$^{-/-}$ thymi into WT mice was sufficient to induce the breakdown in self-tolerance, which is mediated by the loss of Aire function in mTECs (Kajiura et al., 2004; Zhu et al., 2006). In contrast, the impairment of T cell responses in *NIK*$^{aly/aly}$ mice resulted from disrupted NIK signaling in hematopoietic cells (Greter et al., 2009). Also, *NIK*$^{aly/aly}$ mice have a defect in the generation of $T_{reg}$ cells, which is not observed in *NF-κB2*$^{-/-}$ mice (Zhu et al., 2006). These and other observations (Ramakrishnan et al., 2004; Speirs et al., 2004; Zarnegar et al., 2008; Sasaki et al., 2011) suggest that the signaling cascade by which NIK executes its function in cell-mediated immunity may not be exclusive to the p52-dependent noncanonical NF-κB pathway.

As it was widely believed that NIK signaling is predominately involved in the formation of SLTs, the apparent immunodeficiency of the *NIK*$^{aly/aly}$ strain was held as evidence for the requirement of SLTs in the formation of cell-mediated immunity (Hofmann et al., 2010). In recent years, it has however become evident that NIK signaling is also applied by other cell types such as B cells, osteoclasts, cancer cells, and also by DCs and T cells, suggesting a role of noncanonical NF-κB signaling in adaptive immune responses (Matsumoto et al., 2002; Ishimaru et al., 2006; Tamura et al., 2006; Lind et al., 2008; Greter et al., 2009; Jin et al., 2009). We have recently shown that the inability

of *NIK*$^{aly/aly}$ mice to mount cell-mediated immunity is independent of the developmental lymphoreticular malformations but a result of the interrupted NIK signaling in hematopoietic cells (Greter et al., 2009). Yet the mechanistic consequences of lesioned NIK signaling in DCs and T cells remained poorly understood or might have been wrongly interpreted.

Recently, it was reported that *NIK*$^{-/-}$ T cells adoptively transferred into *Rag2*$^{-/-}$ mice failed to develop encephalitogenic properties and to secrete proinflammatory cytokines (Jin et al., 2009). The authors concluded that *NIK*$^{-/-}$ T cells are intrinsically defective and that NIK signaling in T cells is vital for the acquisition of an effector phenotype. This assumption is corroborated by a previous study showing that *NIK*$^{aly/aly}$ T cells secrete reduced levels of IL-2 and GM-CSF (Matsumoto et al., 2002). Indeed, we confirmed a reduction in the secretion of proinflammatory cytokines by *NIK*$^{aly/aly}$ T cells and their failure to homeostatically expand. In addition we found that *NIK*$^{aly/aly}$ T cells were anergic toward their cognate antigen and thus failed to acquire pathogenic properties in the context of autoimmune disease. However, our observation that T cell function was impaired as a result of the loss of NIK in hematopoietic accessory cells and more specifically in DCs challenges the concept of a T cell–intrinsic defect in *NIK*$^{aly/aly}$ mice. Three independent experimental setups demonstrated that the T cell defects were the result of a DC-intrinsic utilization of NIK: first, the presence of *NIK*$^{aly/aly}$ DCs together with WT T cells in vivo was sufficient to significantly diminish EAE development (Fig. 4); second, *NIK*$^{-/-}$ T cells could differentiate into fully functional, autoaggressive T cells when NIK was restored in accessory cells only (Fig. 2); and third, when the expression of NIK was transgenically restricted to DCs via the CD11c promoter, cell-mediated immunity was fully restored even if T cells were *NIK*$^{-/-}$ (Fig. 5). One caveat is that CD11c-Cre was also active in a small population of T cells. This small population of transgenic NIK-expressing T cells could potentially be involved in the rescue of immune function in DC$^{NIK}$-*NIK*$^{-/-}$ → WT BMCs. However, this is most unlikely because (a) we did not observe a preferential accumulation of NIK-expressing GFP$^+$ T cells in the inflamed CNS at peak disease in DC$^{NIK}$-*NIK*$^{-/-}$ → WT BMCs (not depicted), (b) T cell function of mixed *Rag1*$^{-/-}$ + *NIK*$^{aly/aly}$ → *Rag1*$^{-/-}$ BMCs was fully restored even though the entire T cell compartment was NIK deficient, and (c) the transgenic expression of NIK$^{WT}$ in T cells of T$^{NIK}$-*NIK*$^{-/-}$-2d2 → WT BMCs did not render mice EAE susceptible (Fig. S2 B). However, preliminary data suggested that ectopic expression of NIK$^{WT}$ in T$^{NIK}$ mice might affect additional aspects of T cell function.

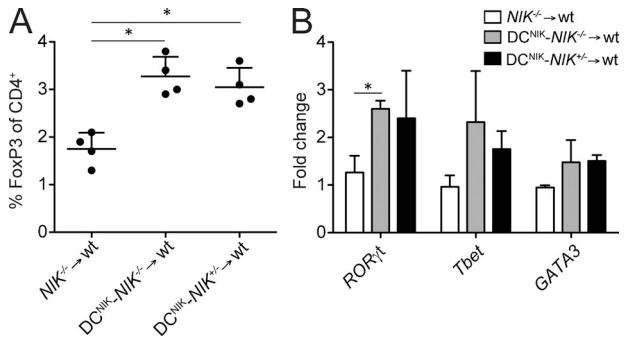


**Figure 8. Restoration of NIK in DCs rescues Foxp3, RORγt, and Tbet expression in developing thymocytes.** (A and B) DC$^{NIK}$-*NIK*$^{-/-}$ → WT, DC$^{NIK}$-*NIK*$^{+/-}$ → WT, and *NIK*$^{-/-}$ → WT BMCs were generated. Percentage of FoxP3$^+$ cells in the thymi (A) and transcript levels of *RORγt*, *Tbet*, and *GATA3* in FACS-sorted CD4$^+$GFP$^-$ SP thymocytes (B) were analyzed. Shown is fold change of expression levels compared with one of three *NIK*$^{-/-}$ → WT samples, which was set to 1. Data are representative of two independent experiments ($n \geq 3$). Error bars indicate SD. *, $P \leq 0.05$.

Interestingly, adoptively transferred adult *NIK*$^{aly/aly}$ T cells failed to acquire pathogenicity but rather displayed an anergy-like state, although they were primed by NIK-sufficient accessory cells (Fig. 1). Only upon undergoing thymic development in the presence of NIK-sufficient accessory cells could CD4$^+$ T cells give rise to pathogenic effector cells. This suggests that against current belief, NIK is largely dispensable within T cells. In contrast, we suggest that the anergic T cell phenotype observed in *NIK*$^{aly/aly}$ and *NIK*$^{-/-}$ mice is caused by defective T cell development caused by dysfunctional thymic DCs. We therefore propose that NIK signaling is critical in DCs to license T cells during thymic development and avoid anergy.

A role for noncanonical NF-κB signaling in DCs has already been suggested, but the precise impact on T cell function has not been addressed before. Although it was claimed that CD40-mediated activation of DCs is not dependent on NIK (Garceau et al., 2000; Yamada et al., 2000; Andreakos et al., 2003), others have demonstrated that peripheral *NIK*$^{aly/aly}$ DCs show reduced APC capacity, which results in diminished T cell proliferation (Tamura et al., 2006). Furthermore, *NIK*$^{aly/aly}$ DCs showed a decreased ability to induce the expansion of CD25$^+$ CD4$^+$ T cells in vitro (Tamura et al., 2006) and were unable to cross-prime CD8$^+$ T cells to exogenous antigen, involving multiple defects in antigen-processing pathways (Lind et al., 2008).

We demonstrate that splenic *NIK*$^{aly/aly}$ DCs produced lower levels of IL-12, IL-23, and IL-6. Furthermore, we show that *NIK*$^{aly/aly}$ DCs were hampered in the priming of CD4$^+$ autoreactive T cells in vivo (Fig. 4). However, the relevance of NIK signaling in thymic DCs has until now not been addressed, most likely because of the incomplete understanding of the function of thymic DCs in general.

Currently, three phenotypically distinct thymic DC subsets have been described, namely pDCs, CD172α$^+$CD11b$^+$ CD8α$^{-/lo}$ migratory cDCs, and CD172α$^-$CD11b$^-$CD8α$^{hi}$ thymic resident cDCs (Wu and Shortman, 2005). One important function of thymic DCs appears to be negative selection at the CD4$^+$ SP stage in T cell development (Brocker et al., 1997; Dakic et al., 2004; Gallegos and Bevan, 2004; Bonasio et al., 2006). In this context, death caused by high-affinity interaction or by no interaction (death by neglect) is not the only fate of developing thymocytes, but also the

generation of $nT_{reg}$ cells or induction of anergic T cells (Ramsdell et al., 1989; Ramsdell and Fowlkes, 1990; Bendelac, 2004). The mechanistic underpinnings of these processes are up to today poorly understood. Although the effect of the complete lack of DCs on T cell development and negative selection is discussed controversially (Birnberg et al., 2008; Ohnmacht et al., 2009), a diverted function of thymic DCs is expected to have consequences on T cell development.

The expression of various chemokine receptors and chemokines in thymic DC subsets has been analyzed and compared with expression profiles of splenic DCs (Proietto et al., 2008a). We also profiled thymic DCs of *NIK*$^{aly/aly}$ mice and found strongly reduced gene expression of *CCR2*, *CCR5*, *CCR6*, and *CCR7*. It has been proposed that these chemokine receptors are important for the localization and migration of DCs into lymphoid tissues in general including the thymus (Bonasio et al., 2006). We further observed a strong reduction in the expression of the chemokines *CCL17*, *CCL19*, and *CCL21* in thymic DCs of *NIK*$^{aly/aly}$ mice, which are important for intrathymic migration of positively selected thymocytes from the cortex to the medulla (Ueno et al., 2004; Proietto et al., 2008a).

Thymic DCs have been proposed to induce FoxP3 expression and the formation of $nT_{reg}$ cells (Proietto et al., 2008b). Recently, it became evident that also other αβ T effector cell lineages such as $T_H17$ cells can at least partially be licensed already during thymic development (Marks et al., 2009). Of note, for γδ T cells, a similar commitment toward IL-17 or IFN-γ production has been shown previously (Ribot et al., 2009), and a very recent study suggested both RelA and RelB to be the critical factors for this process (Powolny-Budnicka et al., 2011). We found that thymic licensing of αβ $CD4^+$ T effector lineages at least to a certain extent relies on the function of NIK and that the expression of $NIK^{WT}$ in DCs alone could rescue not only FoxP3 but also Tbet and RORγt expression in developing NIK-deficient thymocytes. Therefore, we propose that NIK signaling in thymic DCs is crucial to imprint developing T cells to subsequently acquire full effector capabilities and to avoid progression into an anergic state.

## MATERIALS AND METHODS

**Mice and BM reconstitution.** C57BL/6 (WT) and *Rag1*$^{-/-}$ mice were purchased from the Jackson Laboratory and bred in house. Alymphoplasia mice (*Map3k14*$^{aly}$ mice here depicted as *NIK*$^{aly/aly}$) were obtained from Clea Laboratories and bred in house. *NIK*$^{-/-}$ mice on 129Sv/Ev background were provided by R.D. Schreiber (Washington University in St. Louis, St. Louis, MO) and bred onto C57BL/6 background in house for 10 generations. Both *NIK*$^{aly/aly}$ and *NIK*$^{-/-}$ mice were maintained by heterozygous breedings. *NIK*$^{aly/+}$ and *NIK*$^{+/-}$ mice are haplo-sufficient (Miyawaki et al., 1994; Yanagawa and Onoé, 2006), justifying the use of heterozygous animals as littermate controls. Furthermore, *NIK*$^{aly/aly}$ and *NIK*$^{-/-}$ mice are identical in several aspects of their structural and functional impairments (Yin et al., 2001; Greter et al., 2009; Jin et al., 2009). In all breedings with *CD4-cre* and *CD11c-cre* as well as R26$NIK^{WT}$ mice, which are pure C57BL/6, we used *NIK*$^{-/-}$ animals to avoid the usage of mixed genetic backgrounds. Nonetheless, to ensure consistency between the different strains used, we analyzed *NIK*$^{aly/aly}$ and *NIK*$^{-/-}$ mice and heterozygous controls as well as *NIK*$^{+/+}$ mice for expression of effector cytokines and the proportion of $nT_{reg}$ cells (Fig. S4). 2d2 (MOG-TCR transgenic) mice were provided by V. Kuchroo (Harvard Medical School, Boston, MA). *CD11cDTR* mice were provided by S. Jung (Weizmann Institute of Science, Rehovot, Israel). *CD11c-cre* mice were provided by B. Reizis (Columbia University, New York, NY). *R26Stop*$^{FL}$*NIK*$^{WT}$ and all other mice were maintained under specific pathogen-free conditions.

BMCs were generated as described previously (Becher et al., 2002, 2003). In brief, mice were lethally irradiated with a split dose of 1,100 rad. Femur, tibia, and pelvis of donor animals were flushed with PBS to obtain BM stem cells. $10 \times 10^6$ cells were injected i.v. per mouse. Mice were treated with 0.2% BORGAL in drinking water for 3 wk to prevent bacterial infections. All experiments involving animals were approved by the Swiss Cantonal Veterinary Office (13/2006, 55/2009; Zurich, Switzerland).

**Induction of EAE and DTx treatment.** EAE was induced as described previously (Gutcher et al., 2006; Gyülvészi et al., 2009). In brief, mice were immunized s.c. with 200 µg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK; GenScript) emulsified in CFA (Difco) and two i.p. injections of 200 ng pertussis toxin on days 0 and 2. BMCs did not receive pertussis toxin. For EAE experiments with DTx treatment, mice were injected i.p. with 400 ng DTx (EMD) 1 d before immunization and then 200 ng DTx every second day for the entire length of the experiment.

**Isolation of splenic DCs and in vitro stimulation.** Spleens were removed under sterile conditions. Each spleen was injected with a cocktail of 1 mg/ml Liberase and 0.5 mg/ml DNaseI (Roche) in medium and incubated at 37°C for 20 min. Single cell suspensions were prepared by homogenizing the tissue between glass slides and filtering through 70-µm cell strainers followed by erythrocyte lysis. Splenic DCs were isolated with $CD11c^+$ positive magnetic selection according to the manufacturer's instructions (Miltenyi Biotech).

DCs were plated at a concentration of $10^6$ cells/ml in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin-streptavidin (Invitrogen) and stimulated for 6–24 h at 37°C and 5% $CO_2$ with 10 µg/ml α-CD40 (FGK 4.5; BioXCell) and 20 ng/ml IFN-γ (PeproTech). Supernatants were analyzed for IL-6 and IL-12/IL-23p40 using ELISA according to the manufacturer's instructions (BD).

**Isolation of thymic DCs and in vitro co-culture assays.** Thymic DCs were isolated as previously described (Wirnsberger et al., 2009). In brief, thymi were digested in IMDM containing 2% FCS, 25 mM HEPES, 0.4 mg/ml Collagenase D (Roche), and DNase for 40 min at 37°C. Afterward, high-density cells were separated from low-density cells by using a discontinuous Percoll density gradient (ρ = 1.115 and ρ = 1.055; GE Healthcare). After removal from the gradient, cells were washed and stained with antibodies against CD11c, CD8, CD172a, and CD45RA, followed by sorting into migratory DCs ($CD11c^+CD172a^+$), resident DCs ($CD11c^+CD172a^-$), and pDCs ($CD11c^{int}CD45RA^+$) on a FACSAria (BD). Purity was routinely >95%.

For in vitro culture, 20,000 DCs were cultured with 100,000 sorted and CFSE-labeled $CD4^+$ thymocytes or peripheral T cells in the presence of 10 µg/ml $MOG_{35–55}$ and 10 ng/ml IL-7. Analysis was performed after 72 h of culture.

**Peripheral $CD4^+$ T cell purification, in vitro stimulation, and adoptive transfer.** Splenocyte single cell suspensions were prepared as described in Isolation of splenic DCs and in vitro stimulation. $CD4^+$ T cells were purified with $CD4^+$ negative magnetic selection according to the manufacturer's instructions (Miltenyi Biotech). The purity was routinely >95% as confirmed by flow cytometry.

For in vitro stimulations of splenic $CD4^+$ T cells, $3 \times 10^6$ $CD4^+$ T cells/ml were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin-streptavidin. Polyclonal $CD4^+$ T cell activation was performed with 5 µg/ml plate-bound α-CD3 and α-CD28 for 48 h. For antigen-specific $CD4^+$ T cell activation, whole 2d2 splenocytes were stimulated with 20 µg/ml $MOG_{35–55}$ and 5 µg/ml soluble α-CD28 for 48 h.

Supernatants were harvested, and concentrations of IFN-γ, IL-17, GM-CSF, IL-2, and IL-4 were quantified by ELISA according to the manufacturer's instructions (BD).

For adoptive transfer experiments, $2 \times 10^6$ purified splenic $CD4^+$ T cells in PBS were injected i.v. into *Rag1*$^{-/-}$ mice. Homeostatic expansion of T cells in *Rag1*$^{-/-}$ mice was monitored weekly by tail bleeding and flow cytometry, and the percentage of $CD4^+$ T cells of the lymphocyte gate was calculated.

**Flow cytometry.** For cell surface staining, we used the following antibodies: CD11c, $I^{ab}$, CD80, CD86, CD172a, CD45RA, CD4, CD8, IL-12/IL-23p40, Vα3.2, and Vβ11 (BD). Intracellular FoxP3 staining was performed according to the manufacturer's instructions (eBioscience). Cells were incubated with antibodies at the optimal concentration for 20 min at 4°C, and cells were analyzed either on FACS Canto II or LSRII Fortessa (BD). Postacquisition analysis was performed with either FACS Diva or FlowJo (Tree Star) software. Cytofluorometric analysis of CNS-invading lymphocytes has been described previously (Gutcher et al., 2006). For intracellular cytokine staining, cells were treated with GolgiPlug (BD) for the last 4 h of culture. T cells were additionally stimulated with PMA and ionomycin for the last 4 h of culture. After surface staining, cells were permeabilized with Cytofix/Cytoperm (BD) according to the manufacturer's recommendations and stained intracellularly with IL-12/IL-23p40–specific antibody (BD) or FoxP3-, IL-17 (eBioscience)–, and IFN-γ–specific antibody (BD).

**RNA isolation and qRT-PCR.** Total RNA was isolated according to the manufacturer's instructions (RNeasy Mini Plus kit; QIAGEN). RT was performed using random hexamer primers and Moloney murine leukemia virus RT (splenic DCs) or Superscript II (thymic DCs; Invitrogen). cDNA was analyzed by quantitative real-time PCR (qRT-PCR; Bio-Rad Laboratories) in duplicates using SYBR Green PCR Mastermix (Invitrogen) or hydrolysis TAMRA probes (Roche). The expression level of each gene was normalized to *HPRT* or *DNA Polymerase II*. The following primers purchased from Operon Technologies were used: *HPRT* (5′-GACCGGTCCCGTCATGC-3′ and 5′-TCATAACCTGGTTCATCATCGC-3′), *DNA Polymerase II* (5′-CTGGTCCTTCGAATCCGCATC-3′ and 5′-GCTCGATACCCTGCAGGGTCA-3′), *IL-12/IL-23p40* (5′-GACCATCACTGTCAAAGAGTTTCTAGAT-3′ and 5′-AGGAAAGTCTTGTTTTTGAAATTTTTTAA-3′), *IL-12p35* (5′-TACTAGAGAGACTTCTTCCACAACAAGAG-3′ and 5′-TCTGGTACATCTTCAAGTCCTCATAGA-3′), *IL-23p19* (5′-AGCGGGACATATGAATCTACTAAGAGA-3′ and 5′-GTCCTAGTAGGGAGGTGTGAAGTTG-3′), *TLR9* (5′-GCCTTCGTGGTGTTCGATAAGG-3′ and 5′-GAGGTTCTCGAAGAGCGTCTGG-3′), *CCL17* (5′-TACTTCAAAGGGGCCATTCCT-3′ and 5′-GCCTTGGGTTTTTCACCAATCT-3′), *CCL19* (5′-GGCCTGCCTCAGATTATCTGCCAT-3′ and 5′-GGAAGGCTTTCACGATGTTCC-3′), *CCL21* (5′-GGACCCAAGGCAGTGATGGAG-3′ and 5′-CTTCCTCAGGGTTTGCACATAG-3′), *CCR2* (5′-ACAAGCACTTAGACCAGGCCAT-3′ and 5′-AAACTGGGCACTGTTTGC-3′), *CCR5* (5′-ACTGCTGCCTAAACCCTGTCA-3′ and 5′-GTTTTCGGAAGAACACTGAGAGATAA-3′), *CCR6* (5′-TCCATCATCATCTCAAGCCCTACA-3′ and 5′-AGGGGTGAAGAACCCAAAGAACA-3′), *CCR7* (5′-ACCATGGACCCAGGGAAC-3′ and 5′-GGTATTCTCGCCGATGTAGTCAT-3′), and *CCR9* (5′-TGGCTTGTGTTCATTGTGGGCA-3′ and 5′-ATCCATTGACCAGCAGCAGCAA-3′).

**Online supplemental material.** Fig. S1 shows in vitro stimulated splenic DCs and their expression of IL-12/IL-23p40, IL-12p35, and IL-12p19 as described in Fig. 3. Fig. S2 shows the transgenic GFP expression of $DC^{NIK}$-*NIK*$^{-/-}$ → WT BMCs and the EAE score of $T^{NIK}$-*NIK*$^{-/-}$ → WT BMCs. Fig. S3 summarizes the expression of various chemokine receptors of thymic DC subsets as described in Fig. 6 D. Fig. S4 shows a phenotypic comparison of T cells from *NIK*$^{aly/aly}$, *NIK*$^{aly/+}$, *NIK*$^{-/-}$, *NIK*$^{+/-}$, and *NIK*$^{+/+}$ mice, in particular the expression of FoxP3 and effector cytokines. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110128/DC1.

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