NFκB-Inducing Kinase Deficiency Results in the Development of a Subset of Regulatory T Cells, which Shows a Hyperproliferative Activity upon Glucocorticoid-Induced TNF Receptor Family-Related Gene Stimulation

Li-Fan Lu, David C. Gondek, Zachary A. Scott and Randolph J. Noelle

*J Immunol* 2005; 175:1651-1657; doi: 10.4049/jimmunol.175.3.1651

http://www.jimmunol.org/content/175/3/1651
NFκB-Inducing Kinase Deficiency Results in the Development of a Subset of Regulatory T Cells, which Shows a Hyperproliferative Activity upon Glucocorticoid-Induced TNF Receptor Family-Related Gene Stimulation

Li-Fan Lu, David C. Gondek, Zachary A. Scott, and Randolph J. Noelle

CD4⁺CD25⁺ regulatory T cells (Treg) play an important role in maintaining immunologic tolerance. Glucocorticoid-induced TNFR family-related gene (GITR) expressed preferentially at high levels on Treg has been shown to be a key player of regulating Treg-mediated suppression. A recent study reports that NF-κB-inducing kinase (NIK) expression in thymic stroma is important for the normal production of Treg but not for its suppression capacity. In this report, we have shown that Treg from NIK-deficient mice display hyperproliferative activities upon GITR stimulation through an IL-2-independent mechanism. Furthermore, high dose IL-2, anti-CD28 stimulation, or GITR ligand-transduced bone marrow-derived dendritic cells used as APC (culture conditions which drive Treg proliferation in vitro) could not ablate this difference in proliferative activity between NIK-deficient and wild-type Treg. Additional experiments have shown NIK-deficient mice have a higher ratio of CD4⁺CD25⁺cell number than WT mice. The analysis presented herein of Treg subsets in NIK-deficient mice reveals impairment in Treg differentiation. We show that Treg from NIK-deficient mice display a hyperproliferative response upon GITR stimulation. This hyperproliferative response is not mediated by IL-2. Additionally, culture conditions that generally boost Treg proliferation, such as high concentrations of IL-2, anti-CD28 stimulation, or use of GITR ligand (GITRL)-transduced bone marrow-derived dendritic cells (BMDC) as APC, could not ablate this altered proliferative activity. Finally, we show an increased proportion of CD4⁺CD25⁺CD62L⁺ Treg in NIK-deficient mice, and it is this Treg subset that is hyperproliferative to GITR signaling. The significance of this finding and its association to the autoimmunity in NIK-deficient mice is discussed.

Department of Microbiology and Immunology, Dartmouth Medical School and the Norris Cotton Cancer Center, Lebanon, NH 03756

Received for publication January 20, 2005. Accepted for publication May 23, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grants CA91436-01.

2 Address correspondence and reprint requests to Dr. Randolph J. Noelle, Department of Microbiology and Immunology, Norris Cotton Cancer Center, Dartmouth Hitchcock Medical Center, Lebanon, NH 03756. E-mail address: rjn@dartmouth.edu

3 Abbreviations used in this paper: Treg, regulatory T cell; T eff, effector T cell; NIK, NF-κB-inducing kinase; GITR, glucocorticoid-induced TNFR family-related gene; IKK, IκB kinase; BMDC, bone marrow-derived dendritic cell; GITRL, GITR ligand; TU, transducing unit; MOI, multiplicity of infection; WT, wild type; AIRE, autoimmunity regulator.
Materials and Methods

Mice

NIK−/− mice on a 129Sv/Ev background were kindly provided by Dr. R. D. Schreiber (Washington University, St. Louis, MO) (25). In all the experiments, NIK−/− mice were used as control. All mice were maintained by heterozygote mating in a pathogen-free facility.

Abs and reagents

mAb DT-1 to mouse GITR was kindly provided by S. Sakaguchi (Kyoto University, Kyoto, Japan). mAb YGL386 to mouse GITRL was kindly provided by R. D. Schreiber (Washington University, St. Louis, MO) (25). In all experiments, NIK−/− mice were used as control. All mice were maintained by heterozygote mating in a pathogen-free facility.

Lymphocyte purification and proliferation assays

Single-cell suspensions were prepared from 8- to 10-wk-old mice and CD4+CD25− and CD4+CD25+ T cell subsets were purified by magnetic separation according to the manufacturer’s instructions (Miltenyi Biotec). Enriched cell populations and purified cells were phenotypically analyzed by flow cytometry analysis after 48 h. Typical viral preparations yielded 5×10^6 transducing units (TU)/ml. We determined GFP or GITRL antisense activity and suppression was extinguished upon GITR stimulation.

Hyperproliferation of Treg cells in NIK-deficient mice

A recent paper has shown that there is a reduced frequency of Treg in the alymphoplasia mouse (Alv), a strain of mouse with a natural mutation of the NIK gene. While reduced numbers of Treg were observed, the AlvAlv Treg retained their normal suppressive capacity in vitro on a per cell basis (24). Because NIK defect results in the absence of lymph node, we have determined the percentage of Treg in thymus and in spleen. Consistent with this finding, Fig. 1A illustrates that mice in which NIK has been deleted (NIK−/−) have a 50–75% reduction in the ratio of CD4+CD25+ T cells to total CD4 single-positive thymocytes or CD4+ T cells from spleen. Phenotypic analysis of Treg from NIK−/− or NIK+/− mice shows similar CD4+CD25+ populations. However, the expression of CD45RB, CD62L, and CD69 was distinctive in NIK−/− Treg when compared with the expression pattern in NIK+/+. In contrast to the Treg from NIK−/− mice, the majority of NIK+/− Treg were CD45RBhigh, CD62Lhigh but CD69low. We then asked whether the quantitative and phenotypic differences between Treg from NIK−/− or NIK+/− mice would change their regulatory function. As shown in Fig. 1C, the suppressive activity of Treg isolated from either NIK−/− or NIK+/− mice are indistinguishable, as measured by a standard in vitro suppressor assay with different ratios of CD4+CD25−/CD4+CD25+ T cells. Taken together, NIK-deficient mice, like NIK mutant mice, have a reduced number of Treg with comparable suppressive activities.

Hyperproliferation of Treg cells in NIK-deficient mice upon Gitr activation

Previous studies have shown that Gitr signaling not only ablates Treg-mediated suppression, but also provides costimulatory signals to both effector and Treg populations (16−18). Because NIK plays a central role in the signaling of many TNFR family members, the role of NIK deficiency in Gitr-induced Treg stimulation and suppressive activities was evaluated.

NIK function is not involved in Gitr expression as both Treg from NIK−/− and NIK+/− mice express similar levels of Gitr (Fig. 1B). Furthermore, anti-CD3 induction of Gitr on CD4+CD25− T cells was identical in both strains of mice (data not shown). To investigate the potential functional involvement of NIK in Gitr signaling, anti-Gitr-induced ablation of Treg suppression was evaluated. As shown in Fig. 2A, Treg proliferation was suppressed by both NIK−/− and NIK+/− Treg in a standard suppression assay. Treg from NIK−/− mice retained normal suppressive activity and suppression was extinguished upon Gitr stimulation. Treg from NIK+/− mice were anergized or hypoproliferative to Gitr activation in vitro as has been previously shown for wild-type (WT) Treg. In contrast, Treg from NIK−/− mice proliferated in response to Gitr stimulation (Fig. 2A) (14). To confirm Treg proliferation, we purified and CFSE labeled Treg from either NIK+/− or NIK−/− mice and then cocultured with T cell-depleted splenocytes (APCs) for three days with or without Gitr stimulation. Only Treg from NIK−/− mice proliferated vigorously in response to Gitr stimulation.
to GITR activation (Fig. 2B). Moreover, these GITR-mediated hyperproliferative activities were consistent even when the lower concentrations of anti-GITR were used (Fig. 2C). These results demonstrate that NIK+/− Treg have distinctive characteristics from NIK−/− Treg, in that they display a hyperproliferative response upon GITR stimulation while their suppressive capacities remain intact.

**GITR-mediated hyperproliferation is IL-2 independent**

Given that exogenous IL-2 can break Treg anergy in vitro (28, 29), it is possible that T<sup>reg</sup> from NIK-deficient mice produce high levels of IL-2 upon GITR activation to facilitate T<sup>reg</sup> proliferation. IL-2 production from anti-CD3 activated, NIK+/− and NIK−/− CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T cells was measured. As reported in previous studies using A/J mice (21), CD4<sup>+</sup>CD25<sup>+</sup> T<sup>reg</sup> cells have reduced capacity to produce IL-2 in NIK−/− mice in comparison to NIK+/− cells upon anti-CD3 activation. Interestingly, signaling through GITR further increased the IL-2 secretion in both groups (Fig. 3A). As for the CD4<sup>+</sup>CD25<sup>+</sup> population, results were also consistent with previous studies that demonstrate undetectable levels of IL-2 production in both NIK+/− and control cultures (Fig. 3A) (28, 29).

Next, we examined the effect of exogenous IL-2 on T cells from NIK-deficient mice. Although exogenous IL-2 broke the unresponsiveness of T<sup>reg</sup> from both groups as previously shown (28, 29), we have found that both CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> populations from NIK−/− but not from NIK+/− mice, proliferated more extensively in response to GITR stimulation. (Fig. 3B). Taken together, these data suggest that the GITR-mediated hyperproliferative activity of T<sup>reg</sup> in NIK−/− mice is not IL-2 dependent.

**Enhanced proliferation by NIK−/− T<sup>reg</sup> is induced by GITRL-transduced BMDC**

To confirm that engagement of GITR on NIK−/− T<sup>reg</sup> results in enhanced proliferation, GITRL was overexpressed on DCs and the proliferation of T<sup>reg</sup> from WT and NIK−/− mice were evaluated. A lentiviral construct containing a full length GITRL sequence was used to transduce BMDC. The expression levels of GITRL were confirmed by FACS (data not shown). These transduced BMDC were then used as APCs. It is known that in the presence of anti-CD3, DCs can induce the proliferation of T<sup>reg</sup> in the absence of added cytokines. This phenomenon is believed to be dependent upon B7 costimulation by DCs (30). Consistent with this finding, BMDCs transduced with GFP control virus induced T<sup>reg</sup> proliferation from both groups (Fig. 4A). However, overexpression of GITRL resulted in enhanced proliferation of T<sup>reg</sup> from NIK−/− mice but not T<sup>reg</sup> from WT mice. Although GITRL expression was significantly higher after transduction, to rule out the interference of endogenous GITRL expressed by BMDCs, similar results were obtained by using an APC-free system with plate-bound anti-CD3/anti-CD28 plus or minus anti-GITR stimulation (Fig. 4B). These results agree with previous data showing the enhanced proliferation of NIK−/− T<sup>reg</sup> compared with WT T<sup>reg</sup> upon GITR activation.

A higher ratio of CD62L<sup>low</sup> regulatory T cells from NIK-deficient mice is responsible for the enhanced proliferative response to GITR engagement.

CD62L (L-selectin) has been used to discriminate different subsets of T<sup>reg</sup> (31–33). Both CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>high</sup> and CD4<sup>+</sup>C

![Figure 1](http://www.jimmunol.org/)
D25⁺CD62Llow T cells have shown to be suppressive in vitro (32, 34). However, in vivo studies have indicated that only the CD62Lhigh Treg subset and not CD62Llow Treg is suppressive (31, 33). As mentioned earlier, analysis of the Treg populations in NIK⁻/⁻ vs NIK⁺/⁻ revealed a marked reduction of the CD62Lhigh population in NIK⁻/⁻ mice. In thymus, ~50% of the CD4 single-positive and CD25-positive thymocytes from NIK⁺/⁻ mice were CD62Lhigh, whereas only 36% of the Treg from NIK⁻/⁻ mice

FIGURE 2. Treg from NIK-deficient mice displayed a hyperproliferative response upon GITR stimulation. A, CD4⁺CD25⁺ T cells isolated from NIK⁺/⁻ or NIK⁻/⁻ were cocultured with same number of CD4⁺CD25⁺ T cells and irradiated T-depleted APC from NIK⁺/⁻ in the presence of 5 μg/ml anti-CD3 plus 10 μg/ml anti-GITR or RatIg as control in a standard suppression assay as previously described. B, CD4⁺CD25⁺ T cells isolated from NIK⁺/⁻ or NIK⁻/⁻ were CFSE labeled and cocultured with irradiated T-depleted APC for 72 h in the presence of 5 μg/ml anti-CD3 plus 10 μg/ml anti-GITR or Ratlg as control. Cell proliferation was accessed by CFSE dye dilution and FACS analysis. C, CD4⁺CD25⁺ T cells isolated from NIK⁺/⁻ or NIK⁻/⁻ were cocultured with irradiated T-depleted APC for 72 h in the presence of 5 μg/ml anti-CD3 plus different concentrations of anti-GITR and were pulsed with 1 μCi/well [³H]thymidine for the last 8 h of culture. The level of proliferation was measured by [³H]thymidine incorporation.

FIGURE 3. GITR-induced hyperproliferation is not IL-2 dependent. A, CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells from NIK⁺/⁻ or NIK⁻/⁻ were cocultured with APC in the presence of 5 μg/ml anti-CD3 plus 10 μg/ml anti-GITR or Ratlg as control. Supernatants were collected at the indicated times and IL-2 was quantified by ELISA. (N.D., not detected) B, IL-2 (100 U) was added to the standard suppression assay previously described. Proliferation was accessed by [³H]thymidine incorporation.
suppression assay with a 1:1 ratio of CD4+/CD25−/CD4+/CD25+ T cells as previously described. B, The same amount of CD4+/CD25− and CD4+/CD25+ T cells from NIK+/− or NIK−/− were cultured in 96-well U-bottom plate precoated with 1 μg/ml anti-CD3/10 μg/ml anti-CD28 plus 10 μg/ml anti-GITR or Rat Ig as control. (*, p < 0.03).

FIGURE 4. Overexpression of GITR/L on BMDC results in heightened proliferation of NIK−/−, but not WT Treg. A, BM cells were grown in complete RPMI 1640 with GM-CSF and IL-4. On day 5, GFP or GITR lentivirus (MOI of 20) was added into the culture respectively. On day 7, cells were collected, washed, and counted. Some cells were taken to confirm the transduction efficiency; others have been used as APC in a standard suppression assay with a 1:1 ratio of CD4+/CD25−/CD4+/CD25+ T cells as previously described. B, The same amount of CD4+/CD25− and CD4+/CD25+ T cells from NIK+/− or NIK−/− mice were hyperproliferative in response to GITR engagement, NIK deficiency does not, however, alter the suppression mediated by Treg on a per cell basis.

Discussion

The data presented show that NIK plays a pivotal role in regulating the development and expansion of Treg. NIK deficiency results in a diminished representation of the CD62Lhigh Treg subset, and the hyperproliferative response of the CD62Llow subset following GITR signaling. NIK deficiency does not, however, alter the suppression mediated by Treg on a per cell basis.

It is known that Treg are anergic in vitro, but can proliferate upon Ag immunization in vivo (35, 36). In addition to signaling via the TCR, activation through a number of costimulatory molecules such as CD28/B7, 4-1BB/4-1BBL, OX40/OX40L, and GITR/GITRL also regulate Treg anergy and proliferation (18, 37–39). In this study, we show that Treg from NIK+/− mice were hyperproliferative to GITR triggering. Like other TNFR family members, GITR recruits several different TNFR-associated factors to its cytoplasmic tail after engagement of its ligand. GITR signals via these adaptor proteins to mediate the activation of multiple signaling pathways including NIK/NFκB and ERK, which in turn phosphorylate and activate downstream transcription factors (17, 40, 41). It appears that NIK tempers the signaling via GITR, such that in its absence, signaling through GITR induces greater levels of proliferation. These observations may be explained by a recent study by Wallach et al. (42) in studies of CD27 signaling (42, 43). These investigators suggested a possible functional role of interaction between NIK and Siva, a proapoptotic protein, which binds

FIGURE 5. NIK-deficient mice have higher frequency of CD4+/CD25−/CD62Lhigh Treg, which accounts for the hyperproliferative response. A, CD4 single-positive, CD25+ thymocytes, and CD4+/CD25+ spleenocytes from NIK+/− or NIK−/− were stained with CD62L to determine the relative frequency of CD62L high and low cells. The numbers shown in the figure are percent CD62Lhigh positive. CD4+/CD25+ T cells from NIK+/− spleen were then sorted into CD62Lhigh and CD62Llow populations. B, CD62Lhigh and CD62Llow Treg subsets from NIK−/− were tested their proliferative capacity in response to anti-CD3 ± anti-GITR. Those cells were used for [3H]Tdr incorporation assay as mentioned above. C, The suppressive capacities of CD62Lhigh and CD62Llow Treg subsets were tested in vitro, respectively, using culture conditions noted in Fig. 2. D, CD62Lhigh and CD62Llow Treg subsets from NIK−/− were stained for Foxp3 expression according to manufacturer’s instruction.
The involvement of NIK in a wide spectrum of signaling pathways in many different cell types has made it extremely difficult to clearly delineate the basic mechanisms responsible for autoimmunity in NIK-deficient mice. Among the potential consequences of NIK deficiency, dysregulation of AIRE (autoimmune regulator) in NIK$^{−/−}$ mice may play an important role. AIRE has been shown to be a key mediator in the establishment of central tolerance by regulating ectopic expression of tissue-specific Ags in thymus (45).

The involvement of NIK in the development of autoimmune disease is complex, impacting different cell types and different signaling pathways. Taken together, our studies and those published illustrate that NIK deficiency results in the escape of autoreactive T cells to the periphery due to impaired AIRE expression. To compound the impact of heightened peripheral autoreactive T cell activities, NIK deficiency results in the reduced development of Treg, and a skewing of the Treg population from CD62L$^{+}$ to CD62L$^{−}$ resulting in a less efficient and vigorous regulatory T cell response. Moreover, GITR signaling in the periphery would further aggravate the imbalance of CD62L$^{+}$ and CD62L$^{−}$ subsets leading to autoimmunity (Fig. 6).

**Acknowledgments**

We thank Kathy Bennett for great care of mice. We also thank Gary Ward and Alice Givan for cell sorting. The Trono Laboratory (University of Geneva, Geneva, Switzerland) kindly provided the pWPT-GFP lentivirus construct.

**Disclosures**

The authors have no financial conflict of interest.
References


