NF90 regulates inducible IL-2 gene expression in T cells

Lingfang Shi,1 Wayne R. Godfrey,2 Joseph Lin,1 Guohua Zhao,1 and Peter N. Kao1

1Division of Pulmonary and Critical Care Medicine, Stanford University Medical Center, Stanford, CA 94305
2Cellular Immunology, Dendreon Corp., Seattle, WA 98121

Activation of T cells induces the production of T cell growth and survival factor interleukin (IL) 2. Regulatory T cells intrinsically fail to induce IL-2 expression upon activation and can suppress IL-2 production in conventional T cells. Thus, the control of IL-2 expression is critically important to T cell immune responses, yet the mechanisms remain incompletely understood. Nuclear factor (NF) 90 is a zinc-finger DNA- and double-stranded RNA-binding protein subunit that binds specifically to the antigen receptor response element (ARRE)/NF of activated T cells target sequence in the IL-2 proximal promoter. Inducible binding of NF90 to the IL-2 promoter in vivo is shown by chromatin immunoprecipitation. NF90 gene-targeted mice exhibit perinatal lethality. Compared with newborn NF90+/+ mice, newborn NF90−/− mice demonstrate severe impairment of IL-2 expression. Compared with wild-type cells, T cells deficient in NF90 are impaired in ARRE and IL-2 transcriptional activation and IL-2 mRNA stabilization. Fetal liver cells from NF90 gene-targeted mice were transplanted into irradiated adult recombination activating gene (RAG)−2−/− and IL-2Rγ−/− mice deficient in T cells, B cells, and natural killer cells. NF90+/+− and NF90−/−RAG chimeric mice showed grossly normal repopulation of the thymus and spleen, but only NF90−/− T cells were severely impaired in IL-2 gene expression. Compared with littermates, NF90−/− RAG chimeric mice exhibited profound T cell lymphocytopenia in the peripheral circulation. Thus, NF90 regulates inducible IL-2 transcription, mRNA stability, and gene expression in T cells and represents a novel therapeutic target for the modulation of T cell immune responses.
regulator complex (16, 17). The ARRE site was proposed as key for regulating chromatin melting and providing concerted access of all other transcription factors to the IL-2 promoter (18). This model predicts that targeted disruption of the genes that encode critical regulators operating at the purine-box/NFAT site in the IL-2 promoter may confer phenotypes of defective IL-2 transcription and T cell activation and may exhibit T cell immunodeficiency in vivo.

To elucidate the roles of NF90 in development and immune regulation, we generated mice with a targeted disruption of NF90. NF90−/− mice die within 6 h of birth because of diaphragmatic respiratory failure related to insufficient expression of the myogenic regulators MyoD, myogenin, and p21Cip1/Waf1 (5). To circumvent the perinatal lethality of the NF90-deficient mice, we transplanted NF90 fetal liver cells into irradiated recombination activating gene (RAG)−2−/−/IL-2Rγ−/− mice that lack T cells, B cells, and NK cells and characterized immune reconstitution and function in the NF90-RAGγ chimeric mice. In this report, we demonstrate that NF90 inducibly binds to the IL-2 promoter and regulates ARRE/NFAT and IL-2 transcriptional activation, IL-2 mRNA stability, IL-2 gene expression, and peripheral T lymphocyte survival in vivo.

RESULTS AND DISCUSSION

NF90 binds specifically to the IL-2 promoter in vivo in activated T cells

We used chromatin immunoprecipitation (ChIP) to characterize in vivo binding of NF90 to the IL-2 proximal promoter, to IL-2 intron 3 (2.5 kB removed), and to the ADA origin of DNA replication to which Ku80 and Ku70 specifically bind in vivo (Fig. 1) (19). Primary mouse spleen cells were nonstimulated or stimulated for 4 h with PMA + ionomycin (PMA/Iono) and treated with 1% formaldehyde to cross-link transcription factors to their chromatin targets in vivo. Genomic PCR of sheared and restricted input chromatin demonstrated equal amplification of all targets from nonstimulated and stimulated cells (Fig. 1 A). We performed NF90 ChIP using a monoclonal antibody and showed specific binding to the IL-2 proximal promoter in vivo that increased upon stimulation (Fig. 1, B, lane 2 vs. lane 1), and no binding to IL-2 intron 3 (Fig. 1 B, lanes 3 and 4). NF90 bound equally to the ADA origin of DNA replication in nonstimulated and stimulated spleen cells (Fig. 1 B, lanes 5 and 6). Control antibody against hemagglutinin (HA) epitope did not precipitate the IL-2 promoter, intron 3, or ADA chromatin targets in our experimental conditions (Fig. 1 C). This ChIP result validates our original purification using EMSA that identified NF90 and NF45 as subunits of an inducible ARRE/NFAT DNA-binding complex in the nucleus of Jurkat T cells (1).

NF90 genotype determines phenotype of maximal IL-2 gene expression

Adult NF90 heterozygous mice are indistinguishable from wild-type NF90 littermates in terms of size, activity, and longevity.

![Figure 1. NF90 binds specifically to the IL-2 promoter in vivo in activated T cells.](image)

We examined whether adult NF90 heterozygous mice might express less IL-2 than wild-type littermates. Compared with NF90+/+ mice, thymocytes and splenocytes from NF90+/− mice stimulated with PMA/Iono for 6, 12, and 18 h produced ~50% less IL-2 (Fig. 2 A). We also characterized maximal IL-2 gene expression by newborn NF90+/+, NF90+/−, and NF90−/− littermates (Fig. 2 B). Compared with NF90+/+ mice, lymphocytes from NF90+/− and NF90−/− mice stimulated with PMA/Iono for 6 h mice demonstrated moderate and severe impairment of IL-2 expression. The substantial reduction in IL-2 expression in NF90 gene-targeted mice as early as 6 h suggests that expression levels of NF90 regulate transcriptional activation of the IL-2 gene.

**NF90 fetal liver RAG−2−/−/IL-2Rγ−/− chimeric mice characterization**

We transplanted NF90 fetal liver cells, enriched in hematopoietic stem cells, into irradiated adult RAG−2−/−/IL-2Rγ−/− mice that lack T cells, B cells, and NK cells. The resultant chimeric mice developed T and B lymphocytes, allowing us to determine the contributions of NF90 to T cell homeostasis, activation, and IL-2 gene expression.

We performed four independent NF90 fetal liver transplantation experiments that generated 21 NF90-RAGγ chimeric mice. A total of 10 NF90−/−/RAGγ animals were compared with 10 NF90+/− or NF90−/+ RAGγ chimeric littermates. Complete blood counts demonstrated no substantial defects in the reconstitution of erythroid, myeloid, or megakaryocytic lineages. The most consistent defect we observed in NF90−/−/RAGγ chimera was lymphocytopenia (Table I).

**Flow cytometry analyses of T cell development**

NF90-RAGγ chimeric mice were killed 14 wk after transplantation. The thymus and spleens in all NF90-RAGγ chimeric
Figure 2. Targeted disruption of NF90 impairs T cell IL-2 gene expression and T cell homeostasis. (A) Thymocytes and splenocytes were isolated from adult NF90^{+/+} and NF90^{-/-} littermate mice and stimulated with for 6–18 h with PMA/Iono, and secreted IL-2 was measured by ELISA. The data are representative of two independent pairs of littermates. NF90^{−/−} IL-2 reduction was significant (P < 0.05). (B) Thymocytes and splenocytes were isolated from two newborn NF90^{+/+}, two NF90^{−/−}, and one NF90^{−/−} littermate mice and stimulated for 6 h with PMA/Iono, and IL-2 was measured. IL-2 reduction was significant (P < 0.001). Non-stimulated cells produced no detectable IL-2. (C) Immune reconstitution analysis of NF90−/−-RAGγ chimeras. Representative flow cytometry of CD4+ and CD8+ lymphocytes in the thymus and spleen of NF90^{−/−} and NF90^{+/−}/RAG^{−/−} chimeric mice. NF90^{−/−}-RAGγ chimera yielded 10^7 thymocytes, 4.2 × 10^8 splenocytes, and 1,666 peripheral lymphocytes per mm³, and NF90^{+/−}-RAGγ chimera yielded 10^7 thymocytes, 4.5 × 10^8 splenocytes, and 3,455 peripheral lymphocytes per mm³. Recipient mice received 900 rads conditioning radiation and were killed at 14 wk after transplantation with NF90 E14 fetal liver cells. Peripheral blood lymphocyte subset analyses of the same mice at 9 wk after transplantation demonstrated a nearly complete absence of circulating CD4^+ and CD8^+ T cells associated with the NF90^{−/−} genotype (row 3). Spleen cells were gated on CD3 and analyzed for expression of CD4 and CD25 in nonstimulated conditions (row 4) and after 24 h of stimulation with anti-CD3 (row 5). The percentage of cells in each quadrant is shown. (D) Impaired IL-2 secretion in NF90^{−/−}-RAGγ compared with NF90^{+/−} or NF90^{+/+}-RAGγ chimeric mice. Single-cell suspensions of thymocytes, splenocytes, and CD4^+ T cells from individually numbered chimeras were stimulated with PMA/Iono or anti-CD3 + CD28. After 16 h, secreted IL-2 was measured. IL-2 reduction in the NF90^{−/−}-RAGγ chimeric T cells was significant (P < 0.02). Data in A, B, and D represent the mean ± SD.
NF90\(^{−/−}\)/RAG chimeric T cells demonstrate impaired secretion of IL-2

We characterized IL-2 production by NF90-RAG\(γ\) chimeric thymocytes, splenocytes, and purified splenic CD4\(^{+}\) T cells in response to primary stimulations for 16 h with PMA/Iono and anti-CD3 + CD28 (Fig. 2 D). Stimulated NF90\(^{−/−}\)/RAG\(γ\) chimeric T cells (eight mice) consistently demonstrated severely reduced IL-2 expression compared with NF90\(^{+/+}\) or NF90\(^{+/−}\)/RAG\(γ\) T cells (seven littermate mice).

NF90\(^{−/−}\) thymocytes are impaired in ARRE/NFAT transcriptional activation

To address the mechanisms of impaired IL-2 expression in T cells lacking NF90, we characterized transcriptional activation of an ARRE/NFAT luciferase reporter gene transfected into NF90\(^{+/+}\), NF90\(^{+/−}\), and NF90\(^{−/−}\) thymocytes (Fig. 3 A). Compared with NF90\(^{+/+}\) and NF90\(^{+/−}\) cells, NF90\(^{−/−}\) thymocytes demonstrated severe impairment of ARRE/NFAT transcriptional activation upon stimulation (Fig. 3 A).

NF90 transcriptionally activates an ARRE/NFAT luciferase reporter gene in Jurkat T cells

We recently reported that transgenic overexpression of NF45 in Jurkat T cells conferred 120-fold enhancement of IL-2 promoter– and NFAT-luciferase reporter gene activation (20). In this paper we demonstrate that stable transgenic expression of NF90 in Jurkat T cells is associated with 70–80-fold specific enhancement of ARRE/NFAT luciferase reporter gene activation in nonstimulated and PMA/Iono-stimulated T cells (Fig. 3 B). These results indicate that NF90 and NF45 specifically transactivate the ARRE sequence in activated T cells.

NF90\(^{−/−}\) thymocytes are impaired in IL-2 gene transcription

We performed ChIP to examine recruitment and binding of the large subunit of RNA polymerase II to the IL-2 proximal promoter in nonstimulated and stimulated NF90\(^{+/+}\) and
NF90−/− thymocytes. In nonstimulated T cells, we observed no binding of RNA pol II to the chromatin at the IL-2 proximal promoter, consistent with a closed chromatin conformation and no transcription. Wild-type thymocytes stimulated with anti-CD3/CD28 showed prominent induction of RNA pol II binding to the IL-2 promoter, consistent with transcriptional activation (Fig. 3 C, lanes 2 and 4 vs. lanes 1 and 3). In contrast, NF90−/− thymocytes showed substantially reduced binding of RNA pol II to the IL-2 promoter upon stimulation (Fig. 3 C, lanes 6 and 8 vs. lanes 2 and 4).
This result supports our conclusion that NF90 regulates inducible IL-2 transcription.

**NF90 binds to and stabilizes IL-2 RNA**

We used Northwestern blotting to demonstrate that NF90 is a principal IL-2 3′ untranslated region (UTR) RNA-binding protein (Fig. 3 D) (3). Brain extracts are suitable for these studies because NF90 is highly expressed in the brain (5), and IL-2 is expressed in rodent brains (21). A 90-kD protein with the same electrophoretic mobility and IL-2 RNA-binding properties as recombinant NF90 is present in NF90+/− extracts and is absent in extracts prepared from NF90−/− mice. We observed similar results with NF90+/− neonatal thymocytes and a shorter IL-2 RNA probe that contains the four proximal ARREs (unpublished data). We examined IL-2 mRNA stability in splenocytes from newborn NF90+/+ and NF90−/− mice. Upon stimulation with anti-CD3/CD28, NF90+/+ splenocytes prominently induced IL-2 mRNA (Fig. 3 E, lanes 2 and 5 vs. lanes 1 and 4), whereas NF90−/− splenocytes show reduced IL-2 induction (Fig. 3 E, lanes 8 and 11 vs. lanes 2 and 5). Transcription was blocked after 6 h of stimulation by the addition of actinomycin D (22). Compared with NF90+/+ splenocytes, NF90−/− splenocytes showed faster disappearance of IL-2 mRNA after 30 min (Fig. 3 F). Assuming first order exponential decay kinetics, the IL-2 mRNA half-life in NF90+/+ mice is 37 min, whereas the IL-2 mRNA half-life in NF90−/− mice is 12 min. Thus, NF90 binds to and stabilizes newly transcribed IL-2 RNA, thereby posttranscriptionally enhancing IL-2 gene expression.

We propose that NF90 regulates and facilitates rapid expression of IL-2 and other important, tightly controlled genes (including MyoD and p21Cip1) (5). T cell activation triggers IL-2 chromatin remodeling that increases the binding of NF90 and RNA polymerase II to the IL-2 proximal promoter in vivo, as revealed by ChIP. NF90 regulates transcriptional activation through the ARRE sequence in the IL-2 proximal promoter in activated T cells. NF90-deficient thymocytes exhibit specific impairment of ARRE/NFAT-regulated transcriptional activation and binding of RNA pol II to the IL-2 promoter upon stimulation. Binding of newly transcribed IL-2 RNA to NF90 stabilizes the transcript against degradation (3). Operating as an RNA chaperone, NF90 regulates nuclear export of IL-2 RNA and can associate with ribosomes and regulate protein translation (4, 13).

**Flow cytometry.** Engraftment and lymphocyte subsets in peripheral blood were monitored in NF90-RAG chimeric mice at 4 and 9 wk after transplantation. Peripheral blood mononuclear cells were labeled with anti-CD4, -CD8, -CD19, and -DX5 antibodies (Becton Dickinson) and analyzed by flow cytometry (LSR; Becton Dickinson) using FlowJo software (TreeStar Inc.). NF90-RAG chimeras were killed at 15 wk after transplantation. Dissociated thymic and spleen cells were labeled with anti-CD4, -CD8, -CD25, -CD11b, -CD11c, and -B20 antibodies (Becton Dickinson) and analyzed by flow cytometry.

**IL-2 analysis.** Thymocytes, splenocytes, and CD4+ T cells (enriched by magnetic bead negative selection; Miltenyi Biotec) were stimulated (105 cells/ml) for 6–18 h with 20 ng/ml anti-CD3 (Becton Dickinson) + 20 ng/ml anti-CD28 (Becton Dickinson) or 20 ng/ml PMA + 2 μM Iono, and supernatants were analyzed by ELISA (Becton Dickinson) in triplicate.
ARRE/NFAT luciferase reporter gene assays. Primary thymocytes were isolated from newborn NF90+/+ or NF90−/− mice (two littermate animals for each genotype) that function in mRNA processing and interact with the double-stranded RNA-binding motif protein kinase, PKR. J. Biol. Chem. 276:32300–32312.

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BRIEF DEFINITIVE REPORT

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