Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease

Bo Zhu,[1] Alistair L.J. Symonds,[1] Joanne E. Martin,[1] Dimitris Kioussis,[2] David C. Wraith,[3] Suling Li,[4] and Ping Wang[1]

[1]Institute of Cell and Molecular Science, Barts and London School of Medicine and Dentistry, University of London, London E1 2AT, England, UK
[2]Division of Molecular Immunology, Medical Research Council National Institute for Medical Research, Mill Hill, London NW7 1AA, England, UK
[3]Department of Cellular and Molecular Medicine, University of Bristol, School of Medical Sciences, Bristol BS8 1TD, England, UK
[4]Department of Biological Sciences, Brunel University, Uxbridge UB8 3PH, London, England, UK

Maintaining tolerance of T cells to self-antigens is essential to avoid autoimmune disease. How self-reactive T cells are kept functionally inactive is, however, unknown. In this study, we show that early growth response gene 2 (Egr-2), a zinc-finger transcription factor, is expressed in CD44^{high} T cells and controls their proliferation and activation. In the absence of Egr-2, CD44^{high}, but not CD44^{low} T cells, are hyperreactive and hyperproliferative in vivo. The accumulation of activated CD4^{+}CD44^{high} T cells leads to the development of a late onset lupuslike autoimmune disease characterized by the accumulation of interferon (IFN)-γ and interleukin (IL)-17–producing CD4^{+} T cells, loss of tolerance to nuclear antigens, massive infiltration of T cells into multiple organs and glomerulonephritis. We found that the expression of cyclin-dependent kinase inhibitor p21cip1 was impaired in Egr-2–deficient T cells, whereas the expression of IFN-γ and IL-17 in response to T cell receptor ligation was significantly increased, suggesting that Egr-2 activates the expression of genes involved in the negative regulation of T cell proliferation and inflammation. These results demonstrate that Egr-2 is an intrinsic regulator of effector T cells and controls the expansion of self-reactive T cells and development of autoimmune disease.

CORRESPONDENCE
Ping Wang:
p.wang@qmul.ac.uk

Abbreviations used: APC, allophycocyanin; ChIP, chromatin immunoprecipitation; cKO, conditional KO; ds, double stranded; Egr-2, early growth response gene 2.

The engagement of the T cell receptor with antigen can lead to an immune response, tolerance, or homeostatic proliferation, depending on the properties of the antigen and the context in which it is encountered (1). The outcome of a T cell response to antigen stimulation is regulated by the interplay and complex interaction of positive (stimulatory) and negative (inhibitory) pathways. In optimal immune responses, antigen and costimulatory molecules from activated antigen-presenting cells induce strong mitogenic signals in naive T cells, leading to proliferation and differentiation of effector T cells. However, under tolerant or homeostatic conditions, such as the lack of costimulatory signals or self-antigen stimulation, T cells either do not respond or undergo homeostatic proliferation (2, 3, 4, 5, 6). The molecular pathways controlling the different responses after TCR engagement are largely unknown.

Recently, we and others have found that Egr-2 is induced in tolerant T cells in response to antigen stimulation in vivo or TCR ligation in vitro (7, 8, 9). Egr-2 is a member of a family of zinc finger proteins, which consists of four members, Egr-1, -2, -3, and -4, and has been found to play a critical role in hindbrain development and myelination of the peripheral nervous system (10, 11). Together with Egr-1 and -3,

B. Zhu and A.L.J. Symonds contributed equally to this paper.
The online version of this article contains supplemental material.

The Rockefeller University Press $30.00
J. Exp. Med. Vol. 205 No. 10 2295-2307
www.jem.org/cgi/doi/10.1084/jem.20080187

The Journal of Experimental Medicine

Egr-2 is expressed in thymocytes (12) and in mature T cells upon TCR stimulation (7, 8, 9). RNA interference (RNAi)–mediated knockdown of Egr-2 in an established T cell line rendered the cells less susceptible to anergy induction (7), whereas overexpression of Egr-2 reduced T cell activation in vitro (7, 8) indicating that Egr-2 regulates genes involved in the suppression of T cell activation. However, the function of Egr-2 in T cells in vivo has not been studied, as Egr-2 KO mice die perinatally because of defects in hindbrain development (11).

In this study, we demonstrate that Egr-2 is expressed in effector phenotype T cells in the absence of obvious antigen stimulation in vivo. To assess the function of Egr-2 in T cells in vivo, we established Egr-2 conditional KOs (Egr-2 cKO), in which the Egr-2 gene was deleted specifically in $CD2^+$ lymphocytes. The Egr-2–deficient T cells did not show altered primary activation, but were hyperproliferative in response to prolonged stimulation and exhibited a Th1 and Th17 bias leading to the development of a lupuslike syndrome in older mice. Defective expression of p21cip1 was detected in $CD44^{high}$ T cells from Egr-2 cKO mice, and Egr-2 was found to interact directly with the promoter of p21cip1 in vivo. In addition, IFN-γ and IL-17 were highly induced in Egr-2–deficient T cells, and accumulation of IFN-γ– and IL-17–producing cells was associated with massive infiltration of T cells in multiple organs. Our results demonstrate that Egr-2 is important for controlling the self-tolerance of T cells and preventing autoimmunity through activation of negative regulators of cell proliferation and by controlling proinflammatory cytokine expression.

RESULTS

Egr-2 negatively regulates the proliferation of activated T cells, but not naive T cells

In previous studies, we and others found that Egr-2 can be induced in tolerant T cells (7, 8, 9). To study the function of Egr-2 in T cells in vivo, we established Egr-2 cKO by crossing hCD2 promoter-cre transgenic mice (13) with Egr-2-flox/flox mice (14). Egr-2 WT alleles, floxed alleles, and the presence of the hCD2-cre transgene were determined by PCR (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20080187/DC1). Egr-2 was completely deleted from T cells in Egr-2 cKO mice (Fig. 1 A). However, expression of Egr-2 was still detected in B cells from Egr-2 cKO mice (Fig. S1), which is consistent with the weak activity of the CD2 promoter in B cells (13).

The deletion of Egr-2 did not alter the development of the major thymocyte subsets, regulatory T cells, and major subgroups of peripheral T and B cells (Figs. S2 and S3, available at http://www.jem.org/cgi/content/full/jem.20080187/DC1).

In contrast to the suppressive role of Egr-2 discovered by Egr-2 knockdown in a T cell line (7), the activation of Egr-2 cKO T cells in response to primary TCR stimulation was not altered as indicated by normal levels of ERK activation and proliferation (Fig. 1 B and Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20080187/DC1). However, after primary stimulation, under the condition of sustained IL-2 exposure, Egr-2–deficient T cells started to proliferate much faster than T cells from Egr-2-flox/flox mice (Fig. 1 C). To exclude possible effects of the Cre transgene on T cell activation, T cells from hCD2 promoter-cre transgenic mice were also used for stimulation and showed the same responses as T cells from Egr-2-flox/flox mice (unpublished data), which is consistent with previous reports (13). Thus, Egr-2-flox/flox mice were used as a WT control.

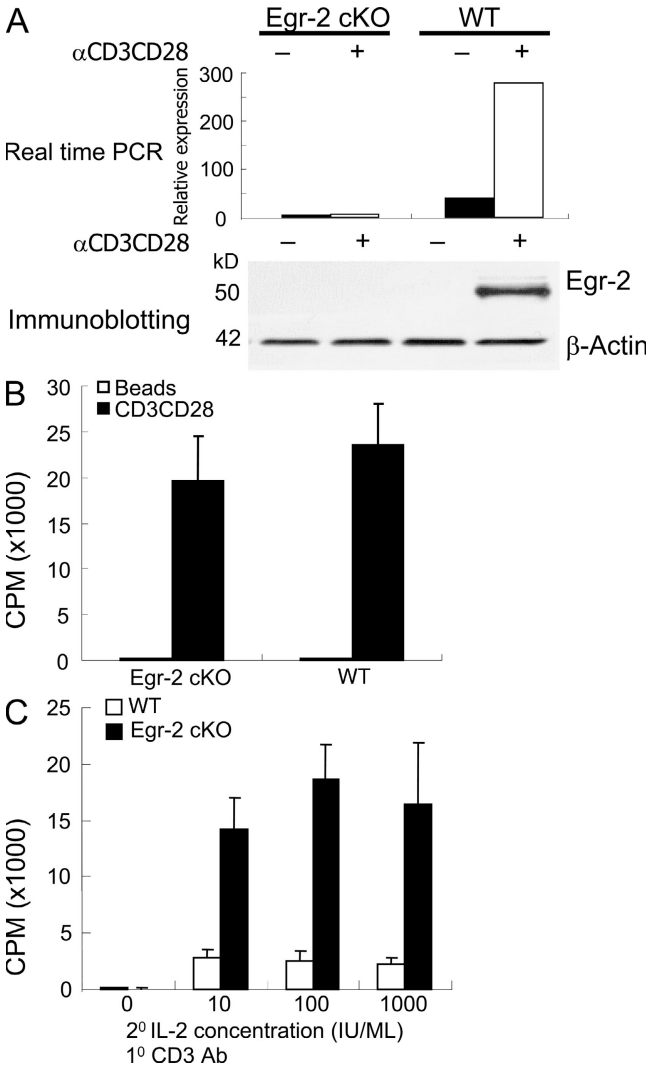

Figure 1. Enhanced proliferative responses of Egr-2–deficient T cells to sustained IL-2 stimulation. hCD2-Cre/Egr-2 floxed mice were identified by PCR with primer pairs specific for iCre, WT Egr-2, and floxed Egr-2 (Fig. S1). (A) The expression of Egr-2 in T cells from spleens of Egr-2 cKO and WT mice before and after TCR ligation in vitro was examined by real-time RT-PCR and immunoblotting. The relative expression of Egr-2 mRNA was measured after normalizing against β-actin. (B) Proliferation of $CD4^+$ T cells from spleens of Egr-2 cKO and WT mice was analyzed in response to anti-CD3/CD28–coated beads or uncoated beads as a control. (C) $CD4^+$ T cells were first stimulated for 48 h with anti-CD3 (1°), and then expanded in 100 U/ml IL-2 for 3 d. The cells were then recounted and restimulated with IL-2 (2°) at different concentrations as indicated. The data are representative of three to five experiments.

The enhanced proliferation of T cells from Egr-2 cKO mice did not result from alteration of IL-2 signaling, as indicated by a normal level of STAT5 activation in Egr-2–deficient T cells in response to TCR ligation (Fig. S4). These results suggest that Egr-2 controls the turnover of effector T cells, rather than primary T cell activation.

Egr-2 has been found to induce expression of FasL in activated T cells (15, 16). However, we could not detect alterations in FasL expression and apoptosis in Egr-2–deficient T cells before or after TCR ligation (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20080187/DC1). Collectively, these data suggest that Egr-2 does not affect TCR signaling or apoptosis induction, but instead controls the subsequent expansion of activated T cells.

Egr-2 is expressed in CD44^{high} T cells and is required for controlling the proliferation and activation of T cells in vivo

The hyperproliferation of Egr-2–deficient T cells in response to sustained, but not primary, stimulation suggests that Egr-2 deficiency may have more profound effects on T cells that have differentiated to an effector phenotype, rather than naive T cells in vivo. In the absence of antigen immunization, CD44^{high} effector phenotype T cells are predominantly the result of exposure to environmental antigen, such as gut flora, or to certain self-antigens (2). Therefore, the responses of CD44^{high} T cells have to be controlled to prevent the development of autoimmune or hypersensitivity reactions (1, 3–6). To investigate Egr-2 expression in CD44^{high} and CD44^{low} T cells, CD4$^+$CD25$^-$CD44^{high} and CD4$^+$CD25$^-$CD44^{low} T cells were isolated from spleens of WT mice by FACS. Egr-2 transcripts were highly expressed in CD4$^+$CD44^{high} T cells in contrast to the minimal expression in the CD4$^+$CD44^{low} population (Fig. 2 A). Furthermore, Egr-2 protein was only detected in CD4$^+$CD44^{high} T cells (Fig. 2 A). These results demonstrate that Egr-2 is expressed in effector phenotype T cells in vivo. To investigate the effect of Egr-2 deficiency on CD44^{high} T cells, we examined their population at 3, 8, and 15 mo of age and their proliferative potential in WT and Egr-2 cKO mice. The percentage of CD44^{high} CD62L^{low} cells, which is characteristic of effector phenotype T cells, was normal at 3 mo, but increased at 8 mo in Egr-2 cKO mice compared with age-matched WT mice (Fig. 2 B). At 15 mo of age, all of the CD4$^+$ T cells from spleens of Egr-2 cKO mice were CD44^{high} and CD62L^{low} in contrast to 21% of CD4$^+$ T cells in WT mice (Fig. 2 B). In contrast to the dramatic accumulation of CD44^{high} T cells in Egr-2 cKO mice, B cells, NK cells, and macrophages were similar in both mouse types (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20080187/DC1). To assess the proliferative potential of these cells in vivo in the absence of experimental immunization, 8-mo-old mice were fed BrdU for 9 d, and then splenic T cells were analyzed for BrdU incorporation. 29.5% of CD8$^+$CD44^{high} and 21.5% of CD4$^+$CD44^{high} T cells from Egr-2 cKO mice had incorporated BrdU (Fig. 3 A). In contrast, the percentage of CD8$^+$CD44^{high} and CD4$^+$CD44^{high} cells that had incorporated BrdU from WT mice was 14.1 and 12.1%, respectively (Fig. 3 A). Less than 5%

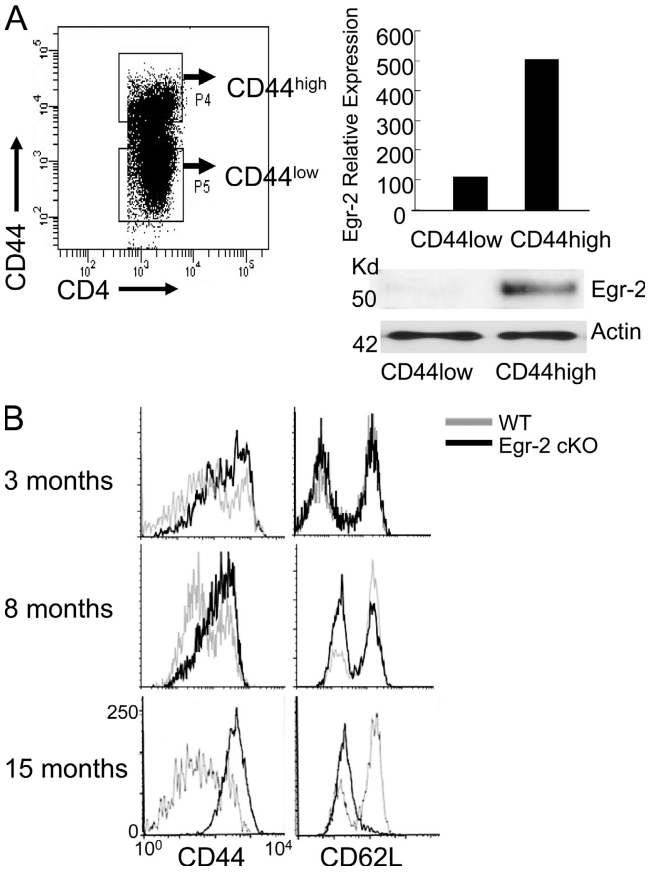

Figure 2. Egr-2 expression and CD44^{high} T cells in vivo. (A) CD4$^+$CD25$^-$CD44^{low} and CD4$^+$CD25$^-$CD44^{high} T cells were sorted from splenocytes of WT mice at 8 mo of age by FACS. The expression of Egr-2 in these two populations was examined by real-time RT-PCR and immunoblotting. The relative expression of Egr-2 mRNA was normalized to β-actin mRNA. The results are representative of three experiments. (B) Splenocytes from Egr-2 cKO and WT mice of different ages were stained for CD4, CD44, and CD62L. Cells were gated on the CD4$^+$ population.

of CD44^{low} T cells incorporated BrdU, and the proportion of BrdU$^+$CD44^{low} T cells was similar in Egr-2 cKO and WT mice (Fig. 3 A). One possible explanation for the hyperproliferation of CD44^{high} cells is that naive CD44^{low} T cells have impaired survival in the absence of Egr-2, and that the resulting lymphopenia induces hyperproliferation of CD44^{high} T cells. To exclude this possibility, CD4$^+$ or CD8$^+$ T cells from 7-mo-old mice were labeled with CFSE and adoptively transferred into WT mice. Consistent with the increase of CD44^{high} T cells in vivo, CD44^{high} T cells from Egr-2 cKO mice proliferated more than CD44^{high} cells from WT mice (Fig. 3 B), indicating that hyperproliferation of CD44^{high} T cells from Egr-2 cKO mice is driven by an intrinsic mechanism.

Consistent with the hyperproliferation of CD4$^+$CD44^{high} T cells, by 15 mo of age, most of the Egr-2 cKO mice developed a spontaneous lymphoproliferative disorder manifesting as splenomegaly, with a spleen size two to five times larger than that of WT mice (Fig. 4 A). The T cell population in

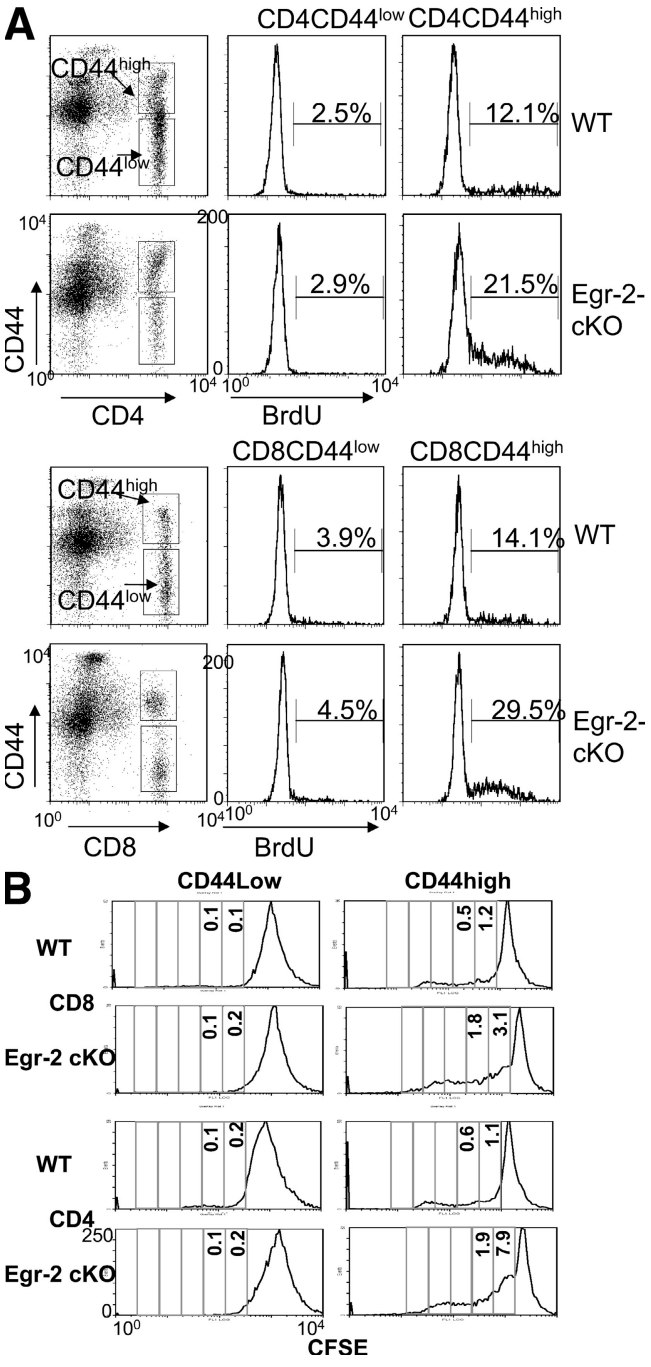

Figure 3. Proliferation of T cells in Egr-2 cKO mice. (A) Mice at 8 mo of age were fed BrdU in drinking water for 9 d. At the end of BrdU treatment, splenocytes were stained with cocktails of anti-CD8, anti-CD44, and anti-BrdU (bottom) or anti-CD4, anti-CD44, and anti-BrdU (top). BrdU-positive T cells were quantified after gating on $CD4^+CD44^{high}$, $CD4CD44^{low}$, $CD8^+CD44^{high}$, or $CD8^+CD44^{low}$ cells. (B). $CD4^+$ or $CD8^+$ T cells were purified from Egr-2 cKO or WT mice at 7 mo of age and labeled with CFSE. The labeled cells were adoptively transferred into syngenic C57BL/6 mice. 3 wk after transfer, splenocytes were stained with anti-CD44. Cells were gated on the $CD44^{high}$ or $CD44^{low}$ population, CFSE dilution was analyzed, and the percentage of cells that had divided was quantified. Data are representative of three experiments.

the spleens of aged Egr-2 cKO mice was significantly increased compared with age-matched WT mice (Fig. 4 A).

In addition to the increase in T cell numbers, most $CD4^+$ and nearly half of $CD8^+$ cells from Egr-2 cKO mice expressed CD25, CD44, and CD69, whereas <20% of $CD4^+$ or $CD8^+$ cells from WT mice did (Fig. 4 B), suggesting that the T cells in aged Egr-2 cKO mice are predominately effector phenotype cells. These results demonstrate that loss of Egr-2 leads to the hyperproliferation and accumulation of activated $CD44^{high}$ T cells in vivo, indicating an intrinsic proliferative disorder of effector T cells.

Development of lupuslike autoimmune disease in Egr-2 cKO mice

We have now provided evidence that Egr-2 plays a central role in the control of effector T cell proliferation and activation in vivo. The absence of Egr-2 resulted in hyperproliferation and accumulation of activated $CD44^{high}$ T cells, suggesting that Egr-2 cKO mice may be more susceptible to autoimmune disease. However, we did not observe any early onset autoimmunity such as diabetes or inflammatory bowel disease in Egr-2 cKO mice younger than 10 mo of age. However, after 1 yr, Egr-2 cKO mice started to show hair loss and became less active. By 15 mo of age, more than half of the Egr-2 cKO mice had died (12 out of 20 mice), whereas no death was observed in age-matched WT mice. In contrast to age-matched WT mice, the majority of the surviving Egr-2 cKO mice exhibited features of systemic lupuslike autoimmune disease. The mice displayed hair loss and skin lesions and an accumulation of inflammatory mononuclear cells in various tissues (Fig. 5, A and B). Importantly, the majority of the infiltrated mononuclear cells were T cells with a smaller proportion of B cells (Fig. 5 C). After 12 mo, Egr-2 cKO mice showed signs of severe glomerulonephritis with high levels of proteinuria (Fig. 5 D), glomerular alterations (Fig. 5 E), and inflammatory cell infiltrates into the kidneys (Fig. 5 B). Kidney histology revealed deposition of immune complexes in the glomeruli of Egr-2 cKO mice, but not WT mice (Fig. 5 F). Although the clinical symptoms of lupuslike disease were only observed in mice older than 12 mo, the glomerular IgG deposits and proteinuria could be detected after 8 mo (Fig. 5 D). We did not observe a difference between the two genders in susceptibility to disease development. To further investigate the lupuslike autoimmunity observed in Egr-2 cKO mice, total serum Ig and Ig subclass distribution was measured in WT and Egr-2 cKO mice aged between 6 and 12 mo. Total serum Ig in Egr-2 cKO mice was increased compared with age matched WT mice (Fig. 6 A). Importantly, among the Ig subclasses, only IgG2a was significantly increased in the sera of Egr-2 cKO mice (Fig. 6 A), indicating a Th1-driven response. Crucially, both anti–double-stranded DNA (dsDNA) and -histone autoantibodies were detected in Egr-2 cKO mice (Fig. 2 B). The titer of autoantibodies against dsDNA and histone increased with age and was closely associated with the accumulation of $CD4^+CD44^{high}$ T cells (Fig. 3 B). In Egr-2 cKO mice aged 2–3 mo, both the percentage of $CD44^{high}$ T cells in the spleen

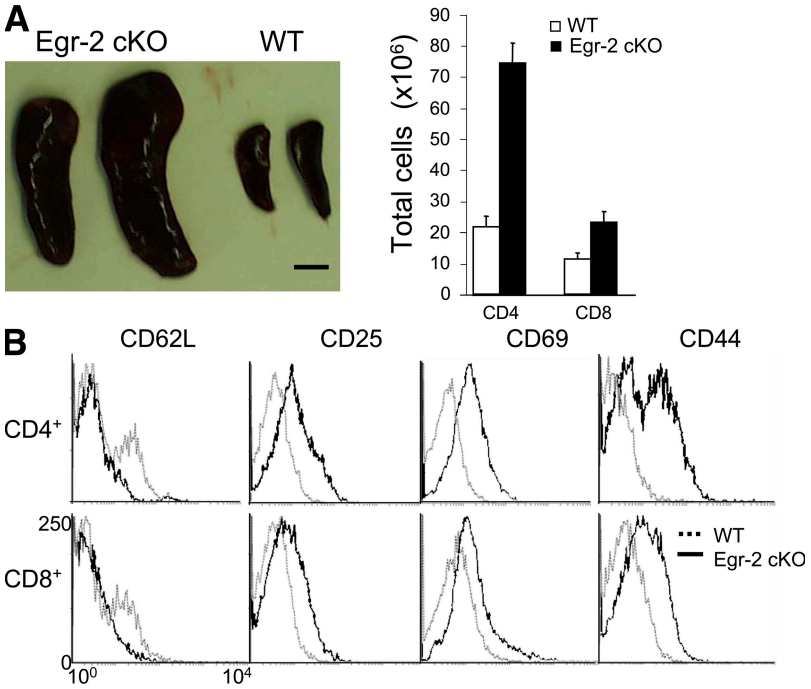

Figure 4. Proliferation and activation of T cells in Egr-2 cKO mice. (A) Spleen size in 15-mo-old Egr-2 cKO and WT mice, and total number of $CD4^+$ and $CD8^+$ T cells in spleens from these mice; presented as mean ± SD. (B) Isolated $CD4^+$ or $CD8^+$ T cells from spleens of Egr-2 cKO and WT mice were stained for surface markers, as indicated. Bar, 5 mm.

and the level of anti-dsDNA and -histone autoantibodies were normal. A low level of autoantibodies was detected after 6 mo, and this increased with age, reaching a very high level in 12-mo-old mice (Fig. 6 B), mirroring the progressive accumulation of activated effector T cells (Fig. 2 B). Disease development was not influenced by the Cre-transgene because anti-dsDNA and –histone autoantibodies were not detected in aged hCD2-Cre transgenic mice, and hCD2-Cre transgenic mice as old as 20 mo displayed a normal phenotype (unpublished data). Thus, Egr-2 deficiency in T cells in mice of the non–lupus-prone C57BL/6 background leads to the loss of self-tolerance and development of an autoimmune disease similar in many respects to human lupus.

The autoimmunity in Egr-2 cKO is T cell dependent

Egr-2 is expressed in activated B cells. We found that the expression of Egr-2 could still be detected in B cells from Egr-2 cKO mice after cross-linking of surface Ig (Fig. S1), suggesting that the weak activity of the CD2 promoter in B cells could not effectively delete Egr-2 (13). The failure to delete Egr-2 in B cells in Egr-2 cKO mice may be the reason for the normal B cell population and minimal B cell infiltration in inflamed tissues (Fig. 5 C, Fig. S1, and Fig. S3), suggesting that T cells are the driving force for the development of autoimmune disease in Egr-2 cKO.

To determine whether the $CD4^+$ T cells in Egr-2 cKO could drive the production of autoantibodies, Egr-2–deficient $CD4^+$ T cells were mixed with T cell–depleted splenocytes from syngenic WT mice, and WT $CD4^+$ T cells were mixed with T cell–depleted splenocytes from Egr-2 cKO mice, and these were then adoptively transferred into $Rag2^{-/-}$ mice of the non–autoimmune-prone C57BL/6 background. 75 d after transfer, anti-dsDNA and -histone autoantibodies were detected in the serum of recipients given the mixture of Egr-2–deficient $CD4^+$ T cells and WT non–T cells (Fig. 6 C), but not in the serum of recipients of WT $CD4^+$ T cells mixed with non–T cells from Egr-2 cKO mice, indicating that $CD4^+$ T cells deficient in Egr-2 are responsible for initiating the development of autoimmune disease.

These results demonstrate that Egr-2 intrinsically controls the tolerance of T cells and prevents the onset of autoimmunity.

Egr-2 activates p21cip1 expression in $CD44^{high}$ T cells

Previously, we found that p21cip1 is induced in the presence of Egr-2 in T cells (9). There is evidence suggesting that p21cip1 is involved in the homeostasis and self-tolerance of T cells (17, 18, 19). Intriguingly, p21cip1 KO mice also develop lupuslike disease (17). In particular, defective p21cip1 expression may be partly responsible for the hyperproliferation of $CD44^{high}$ T cells in Egr-2 cKO mice. To investigate this possibility we examined the expression of p21cip1 in $CD4^+CD44^{high}$ and $CD4^+CD44^{low}$ T cells of WT and Egr-2 cKO mice. We found that the $CD44^{high}$ population expressed approximately fivefold higher levels of p21cip1 than the $CD44^{low}$ population in WT mice (Fig. 7 A). This pattern of p21cip1 expression correlates with that of Egr-2 in WT mice (Fig. 2 A). In contrast, the $CD44^{high}$ population in Egr-2 cKO mice had more than a threefold reduction in expression of p21cip1 compared with WT $CD44^{high}$ cells.

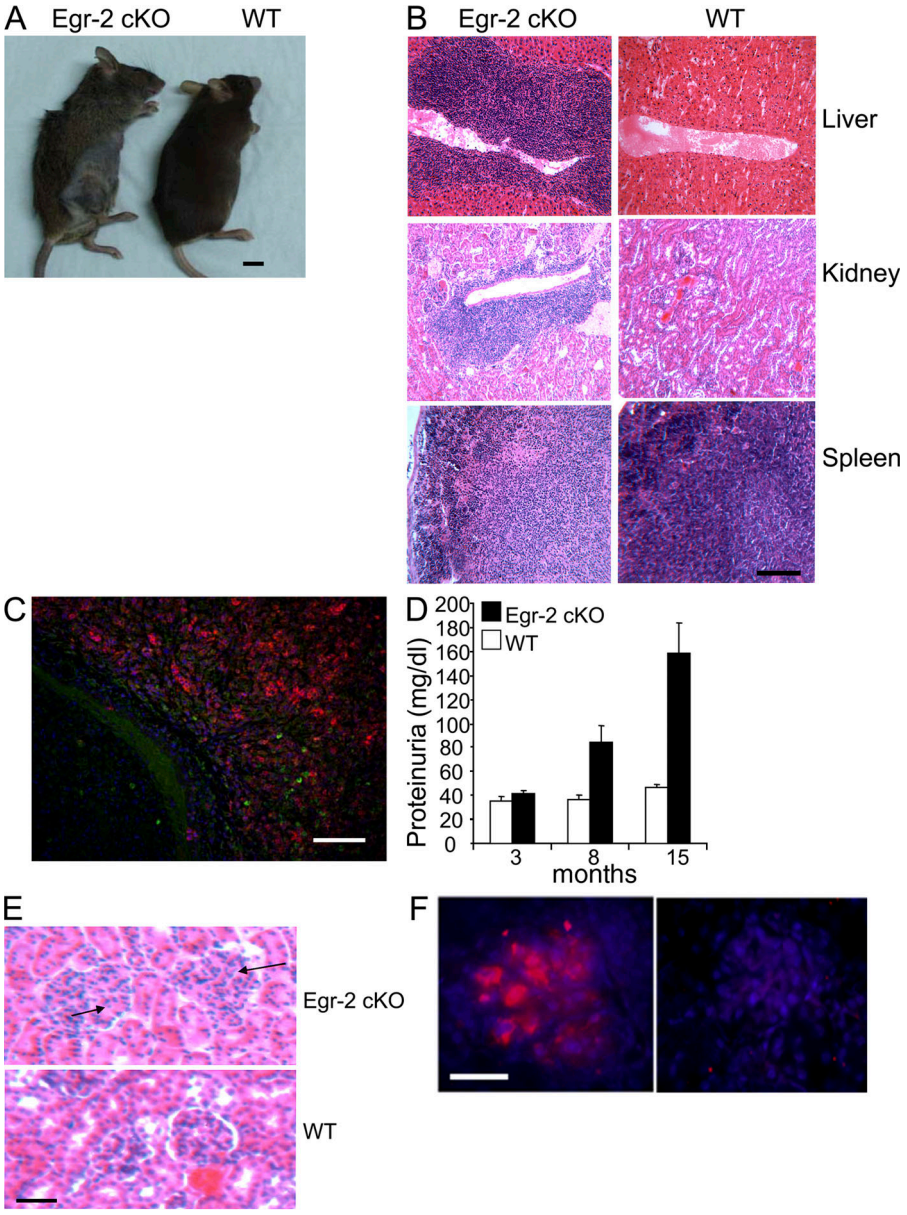

Figure 5. Development of lupuslike autoimmunity in older Egr-2 cKO mice. (A) Skin lesions in 15-mo-old Egr-2 cKO mice compared with age-matched WT mice. (B) Photomicrographs of spleen, liver, and kidney tissues from these mice (×10) stained with hematoxylin-eosin (H-E). Note the expansion of white pulp in spleens and inflammatory infiltrates around the blood vessels of liver and kidney of Egr-2 cKO but not WT. (C) Tissue sections of kidney from Egr-2 cKO mice were stained with antibodies against CD3 (red) and B220 (green). The sections were counterstained with DAPI for nuclear staining. (D) Proteinuria in age-matched WT and Egr-2 cKO mice. Data are the mean ± SD of values from 10 mice/group. (E) Glomeruli of a 15-mo-old Egr-2 cKO mouse (top) and an aged-matched WT mouse (bottom). The arrows indicate typical nuclear fragments. (F) Immune complex deposition in glomeruli of 15-mo-old Egr-2 cKO (left), but not in age-matched WT mice (right) as detected by fluorescently labeled anti–mouse Ig (red). The glomeruli were visualized by DAPI counterstaining in F. Bars: (A) 1 cm; (B) 100 µm; (C) 50 µm; (E) 25 µm; (F) 20 µm.

The expression of p27kip1 was also analyzed, and a similar level of expression was detected in $CD44^{high}$ cells from both WT and Egr-2 cKO mice (unpublished data).

To investigate whether Egr-2 can directly regulate p21cip1 expression we analyzed the 10-kb immediately upstream of the mouse p21cip1 transcriptional start site, a potential Egr-2 binding site (20) was identified within a highly conserved regulatory region of the mouse p21cip1 (−56 to −48) promoter (Fig. 7 B). The p21cip1 promoter contains a GAGGGGGCG site with G residues at the 1, 3, 6, 7, and 9 positions that were shown to be important contacts in the crystal structure of DNA-bound Egr-1 (10), which is the closest homologue to Egr-2 in terms of primary structure. To determine whether Egr-2 binds to this potential site, nuclear proteins were extracted from unstimulated and anti-CD3–stimulated MF2.2D9 T cells. Egr-2 was detected only

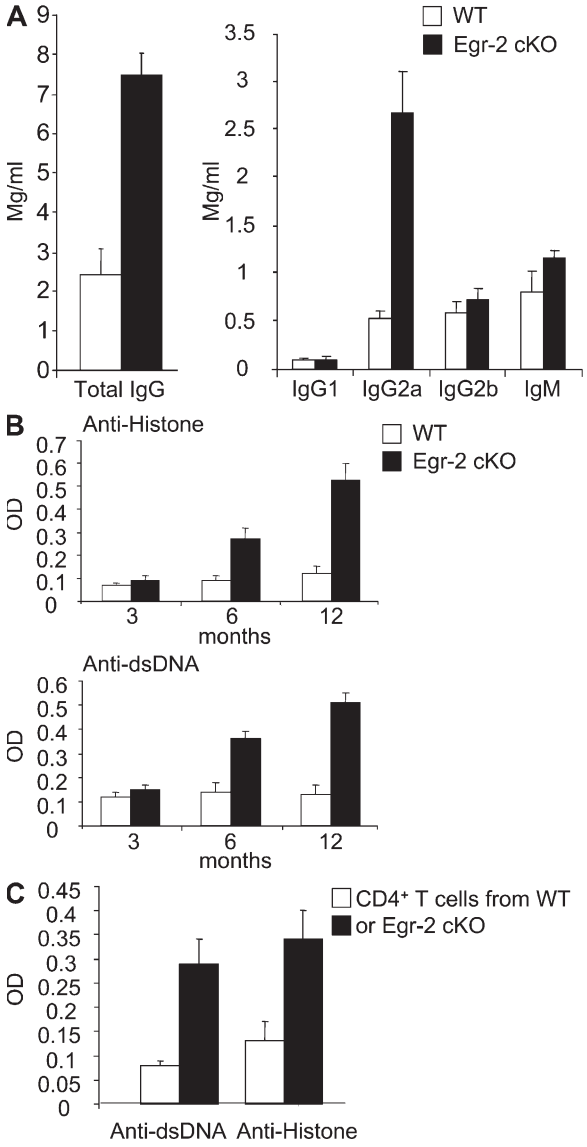

Figure 6. Development of autoantibodies in Egr-2 cKO mice. All the antibodies were measured by ELISA and presented either as milligram/milliliter or absorbance (OD). (A) Serum concentration of total Ig and Ig isotypes. Data represent the mean ± SD, $n = 24$ aged 6–12 mo. (B) Serum concentration of IgG antibodies against dsDNA and histone in age matched Egr-2 cKO and WT mice ($n = 6$/group). (C) Serum concentration of IgG antibodies against dsDNA and histone from RAG $2^{-/-}$ mice that were recipients of T cells from Egr-2 cKO and T cell–depleted splenocytes from WT mice or T cells from WT and T cell–depleted splenocytes from Egr-2 cKO ($n = 5$ mice/group).

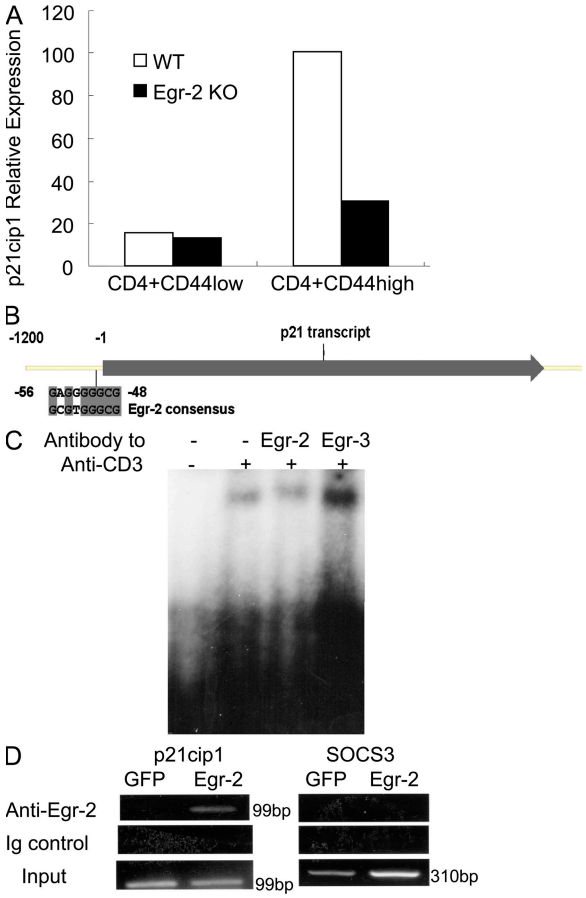

Figure 7. Egr-2 directly activates p21cip1 expression in CD44^{high} T cells. (A) CD4$^+$CD25$^-$CD44^{low} and CD4$^+$CD25$^-$CD44^{high} T cells from WT and Egr-2 cKO mice were isolated by FACS sorting. The expression of p21cip1 was quantified by real-time RT-PCR. The relative expression of p21cip1 mRNA was normalized against β-actin mRNA. The data shown are representative of three experiments. (B) Identification of an Egr-2 binding site in the promoter region of the p21cip1 gene. (C) Electrophoretic mobility-shift assay to evaluate the binding of Egr-2 to oligonucleotides corresponding to this sequence from the p21cip1 promoter. Nuclear extracts from stimulated and unstimulated MF2.2D9 cells were incubated with radiolabeled oligonucleotides, and complexes were resolved by native gel electrophoresis. Supershift was observed with anti-Egr-2 antibody, but not with anti-Egr-3 antibody. (D) Chromatin immunoprecipitation assay. GFP and Egr-2–expressing MF2.2D9 T cells were cross-linked with formaldehyde and chromatin immunoprecipitated with anti-Egr-2 or irrelevant control antibody. The p21 promoter, but not the SOCS3 CDS, was detected in the immunoprecipitates by semiquantitative PCR. Amplification was in the linear range.

after TCR stimulation of MF2.2D9 cells. Nuclear proteins derived from TCR-stimulated, but not unstimulated, MF2.2D9 cells bound to an oligonucleotide corresponding to this site and the specific interaction was confirmed by a supershift induced by anti–Egr-2 antibody, but not Egr-3 antibody (Fig. 7 C). The slow migration of oligonucleotide–Egr-2 complexes suggests that Egr-2 may complex with additional factors. To assess whether Egr-2 protein associates with the proximal regions of the mouse p21cip1 promoter in Egr-2–expressing MF2.2D9 T cells in vivo, a chromatin immunoprecipitation (ChIP) assay was performed using MF2.2D9 T cells transduced with Egr-2 or GFP control retrovirus. The results showed that the p21cip1 promoter was detected in the Egr-2 precipitates by PCR, whereas no signal above background was seen with irrelevant IgG or with primers specific for the SOCS3 gene as controls (Fig. 7 D), indicating that Egr-2 directly binds to the p21cip1 promoter in T cells.

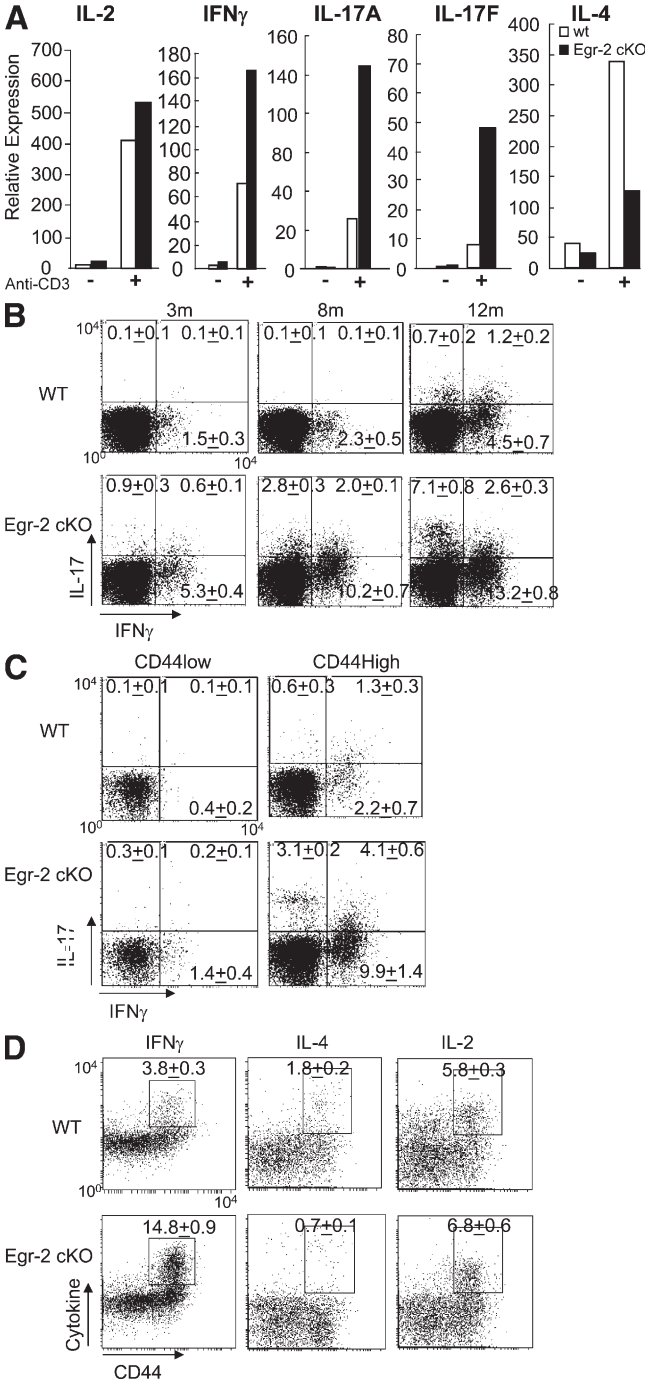

Figure 8. Cytokine expression and T cell differentiation of Egr-2–deficient CD4⁺ T cells. (A) CD4⁺ T cells from 3-mo-old WT and Egr-2 cKO mice were stimulated without (-) or with anti-CD3 (+) for 6 h and cytokine expression was quantified by real-time RT-PCR. The relative expression of cytokines was normalized to β-actin mRNA. The results are representative of three experiments. (B) CD4⁺ T cells from WT and Egr-2 cKO mice of different ages indicated were stimulated for 5 h with PMA and ionomycin before staining with IFN-γ and IL-17. (C) CD4⁺ T cells from 8-mo-old mice were stimulated for 5 h with PMA and ionomycin and then stained with CD44, IFN-γ, and IL-17. The cytokine-positive cells were quantified after gating for $CD44^{low}$ or $CD44^{high}$. (D) Splenocytes from 8-mo-old mice were stimulated for 5 h with PMA and ionomycin and then stained with CD4, CD44, and the indicated cytokine antibodies. Cytokine-positive $CD44^{high}$ cells were quantified after gating for CD4⁺ cells.

Together with the defective expression of p21cip1 in $CD44^{high}$ T cells from Egr-2 cKO mice, these data suggest that Egr-2 directly activates the expression of p21cip1 in $CD44^{high}$ T cells.

T cell differentiation in Egr-2 cKO mice

Although p21cip1 deficiency could explain the proliferative disorder of effector T cells, there may be other mechanisms involved in systemic inflammation. To examine the role of Egr-2 in regulation of effector T cell function, we assessed the expression of cytokines, including IL-2, IFN-γ, IL-4, and IL-17, in Egr-2–deficient and WT CD4 T cells after TCR ligation. These cytokines are signatures for T cell activation, Th1, Th2, and Th17 differentiation, respectively. CD4⁺ T cells were isolated from spleens of 3-mo-old mice to avoid complications from the increased percentage of $CD44^{high}$ T cells in older Egr-2 cKO mice. Consistent with the normal primary responses in vitro (Fig. 1 B), IL-2 was up-regulated normally in Egr-2 cKO cells (Fig. 8 A). However, the expression of IFN-γ, IL-17A, and IL-17F in Egr-2–deficient T cells was much higher than WT (Fig. 8 A). Although IFN-γ was increased approximately twofold in Egr-2–deficient T cells, the increase in IL-17 was most significant with a four- to fivefold increase compared with WT. Interestingly, IL-4 was reduced in Egr-2–deficient CD4⁺ T cells (Fig. 8 A). To investigate the relevance of proinflammatory cytokines to the development of autoimmune disease, IFN-γ– and IL-17–producing T cells, which are the hallmarks of Th1 and Th17, respectively, were analyzed in CD4⁺ T cells from different ages. In WT mice, the percentage of IFN-γ–producing cells (~1.5–2%) was similar in 3- and 8-mo-old mice, whereas IL-17–producing cells could barely be detected at either age (Fig. 8 B). However, a low level of IL-17–producing cells (~1.9%) were detected in WT T cells from mice aged 12 mo, and IFN-γ–producing cells were also increased to ~4.5% in these mice (Fig. 8 B). In contrast, a low level of IL-17– (~1.5%) and IFN-γ–producing (~5%) T cells could already be detected in T cells from young (3-mo-old) Egr-2 cKO mice, and the percentage of cells producing IL-17 and IFN-γ progressively increased as the mice aged (IL-17, ~5% at 8 mo and ~9% at 12 mo; IFN-γ, ~10% at 8 mo and ~13% at 12 mo; Fig. 8 B).

We have shown that Egr-2 deficiency leads to the accumulation of $CD44^{high}$ T cells. To determine whether the expanded $CD44^{high}$ T cells in Egr-2 cKO mice are functionally altered, the function of $CD4^{+}CD44^{high}$ T cells in WT and Egr-2-cKO mice at 8 mo of age was assessed by measuring the proportion of IFN-γ–, IL-2–, IL-4–, and IL-17–producing cells. More than 14 and 7% of $CD4^{+}CD44^{high}$ cells from Egr-2 cKO mice produced IFN-γ and IL-17, respectively, compared with <4 and <2% of $CD4^{+}CD44^{high}$ cells in WT mice (Fig. 8 C). In contrast, <2 and<0.5% of $CD4^{+}CD44^{low}$ cells produced IFN-γ and IL-17, respectively, in either Egr-2 cKO

or WT mice (Fig. 8 C). However, the percentage of IL-4–producing cells was reduced in Egr-2–deficient T cells (Fig. 8 D). Thus, the large population of $CD4^+CD44^{high}$ T cells in Egr-2 cKO mice are predominately Th1 or Th17 type effector T cells. Importantly, the percentage of IL-2–producing T cells was very similar in Egr-2 cKO and WT mice (Fig. 8 D), indicating that the expansion of $CD44^{high}$ T cells in Egr-2 cKO is not caused by enhanced IL-2 expression. These results suggest that hyperproliferation of effector T cells and Th1 and Th17 differentiation are the major disorders in Egr-2 cKO mice.

To investigate the mechanisms behind the increased expression of IFN-γ and IL-17, we examined the expression of T-bet, a Th1 transcription factor, and IL-6, -21, and -23, which can induce Th17 differentiation in $CD4^+$ T cells from aged Egr-2 cKO mice. The expression of these molecules was not altered (unpublished data), suggesting that Egr-2 may be directly involved in regulation of IFN-γ and IL-17 expression or Th1 and Th17 differentiation.

DISCUSSION

In this study, we have discovered that Egr-2 controls the proliferation and tolerance of T cells. Loss of Egr-2 expression leads to the accumulation of $CD44^{high}$ T cells, enhanced expression of proinflammatory cytokines, increased Th1 and Th17 differentiation, and development of a late onset lupus-like autoimmune disease.

We and others have previously found that Egr-2 is induced in tolerant T cells after TCR stimulation (7, 8, 9). We have now found that Egr-2 is normally expressed in $CD44^{high}$ T cells, and that Egr-2 deficiency results in a massive accumulation of these cells. $CD44^{high}$ is a marker for effector or memory T cells (2). The $CD44^{high}$ T cells in WT mice raised in a clean environment are presumably generated in response to commensal antigens, gut flora, or self-antigen (2). In contrast to the resting $CD44^{low}$ naive T cells, $CD44^{high}$ cells undergo slow and intermittent proliferation in response to homeostatic stimuli such as self-antigen and cytokines (2), and also it has been found that $CD44^{high}$ memory T cells are intrinsically more sensitive than $CD44^{low}$ naive cells to stimulation through the TCR–CD8 complex (21). The expression of Egr-2 in $CD44^{high}$ cells may be induced from homeostatic responses to serve as a feedback mechanism to control the proliferation and activation of $CD44^{high}$ T cells. The development of systemic autoimmune responses in Egr-2 cKO mice demonstrates the importance of such controls.

Several findings in predisposed mice appear to support the notion that primary or secondary homeostatic lymphocyte perturbations, such as in MRL/lpr and p21cip1 KO mice of C57BL/6 background, contribute to the pathogenesis of spontaneous lupus (17, 22, 23). These findings suggest that uncontrolled T cell proliferation can lead to T cell expansion and generation of effector cells to self-antigens, resulting in the development of lupuslike systemic autoimmune diseases. Our results indicate that Egr-2 is an intrinsic regulator that controls not only proliferation but also inflammation of effector T cells, and also inhibits the development of lupuslike autoimmune diseases. The accumulation of IFN-γ– and IL-17–producing $CD4^+$ T cells in Egr-2 cKO mice suggests the possibility that the activated $CD4^+$ T cells are effector inflammatory T cells primed by self-antigen, and that the loss of tolerance in Egr-2 cKO mice leads to the expansion of these cells and autoimmune responses.

We found that the cell cycle inhibitor p21cip1 is a direct target gene of Egr-2. Egr-2 deficiency results in defective expression of p21cip1 in $CD44^{high}$ T cells. Most of the reports from $p21cip1^{-/-}$ mice have shown enhanced T cell activation, proliferation, and autoimmune manifestations (17, 18, 19), which are largely consistent with Egr-2 cKO mice. However, a study in the BXSB atypical lupus model showed that p21cip1 deletion increased apoptosis of activated T cells and decreased disease incidence in male mice suggesting that p21cip1 regulates proliferative responses by blocking apoptosis (24). However, the increased apoptosis in response to TCR stimulation suggests a differential effect of p21cip1 in the activation and proliferative responses of T cells. In a recent study in C57BL/6 mice, p21cip1 deletion only enhanced T cell proliferation at a late stage of stimulation (19), which is consistent with our findings in Egr-2 cKO mice of C57BL/6 background. Collectively, these studies indicate that p21cip1 is involved in the regulation of effector T cell proliferation, but plays only a part of the tolerance program, and its function in lupus development is dependent on the genetic predisposition. Indeed, in addition to the altered expression of p21cip1, enhanced differentiation of Th1 and Th17 cells in Egr-2 cKO mice was found. Although we do not yet know how Egr-2 regulates the expression of proinflammatory cytokines, the accumulation of Th1 and Th17 cells can directly result in infiltration of T cells into multiple organs and inflammatory autoimmune diseases (25). These results have not been reported in p21cip1-deficient mice (17).

The increased expression of IFN-γ and IL-17 in Egr-2–deficient T cells after TCR ligation suggests that Egr-2 may be involved in the control of inflammatory cytokines in effector T cells. Such control may not only be important to prevent inflammatory reactions of autoreactive T cells but also for the reduction of immunopathology during a productive immune response. Although both Th1 and Th17 are observed in most autoimmune models, the mechanisms mediating Th1 and Th17 differentiation in vitro are distinct (25), whereas the in vivo mechanisms are still unknown. NFAT has been found to induce IFN-γ expression and activate the IL-17 promoter (26, 27). Egr-2 is one of the NFAT target genes in T cells. Therefore, the expression of Egr-2 could act as negative feedback loop to control IFN-γ and IL-17 expression in response to TCR stimulation. Although we did not find altered activation of NFAT in Egr-2–deficient T cells from aged mice (unpublished data), the possibility that Egr-2 physically interacts with NFAT and inhibits its activity has yet to be analyzed. The reduced production of Th2 cytokines could either result from enhanced Th1 and Th17 differentiation or direct regulation by Egr-2; future investigations will further examine these hypotheses.

Both Egr-2 and -3 are expressed in double-negative thymocytes, and defective expression of Egr-3 leads to the accumulation of double-negative thymocytes and reduction of DP thymocytes in the thymus (12), indicating that Egr-3 is involved in thymocyte development before thymocyte selection. However, we did not find a similar phenotype in the thymus of Egr-2 cKO mice, suggesting that either Egr-2 function at this stage is redundant or that Cre-mediated deletion was incomplete at this stage. T cell lines overexpressing Egr-2 or -3 show an up-regulation of Cbl-b and reduced production of IL-2 (7, 8). This finding led to the hypothesis that the mechanism for Egr-2 in maintaining T cell tolerance would be down-regulation of TCR signaling. However, we could not detect differences between Egr-2–deficient and WT naive T cells in the major TCR signaling pathways, such as AP1, NF-κB, NFAT, and MAP kinase, after TCR stimulation in vitro (Fig. S4 and not depicted). In addition, we did not observe hyperproliferation of naive Egr-2 cKO T cells in response to primary TCR stimulation. This normal response to TCR engagement could be caused by functional compensation by Egr-3 (8). Nevertheless, the continuously proliferating T cell lines (7, 8) and preactivated primary T cells (8) used in these studies share some features of the hyperactive $CD44^{high}$ T cells in Egr-2 cKO mice. Therefore, the negative regulation of T cell activation in these reports is consistent with the hyperproliferation and activation of Egr-2–deficient T cells in vivo.

Egr-2 has been found to induce FasL expression in T cells (15, 16). Deficiency in Fas expression in MRL/lpr mice results in severe lupuslike disease caused by the resistance of T cells to apoptosis (22, 23). However, Egr-2 deficiency did not alter expression of FasL in T cells or apoptosis in either young or old mice, and $CD3^+CD4^-CD8^-$ T cells were not detected in spleen of old Egr-2 cKO mice (Fig. S5), suggesting distinct mechanisms for lupuslike disease in Egr-2 cKO and MRL/lpr mice. Recently, reduced expression of Egr-2 and -3 has been found in T cells from BALB/c mice after induction of lupuslike disease by an anti-DNA antibody, and the decreased expression was associated with increased IFN-γ secretion (28), suggesting that Egr molecules are involved in the maintenance of self-tolerance.

Although antinuclear antibodies are hallmarks of systemic lupus, the mechanisms responsible for the breakdown of self-tolerance are still unknown. One of the characteristics of the autoimmune disorder in lupus patients is Th1-mediated inflammation with a high level of IFN-γ production and increased serum IgG2a antibody (22), both of which we have observed in aged Egr-2 cKO mice. In addition, a massive increase in IL-17 production in activated T cells and an increased percentage of Th17 cells in Egr-2 cKO mice were observed. Th17 plays an important role in the development of lupuslike disease (29). These results suggest that autoimmune disorders can result from a loss of control of effector T cell expansion and inflammatory activation, and that this control is mediated by genes regulated by Egr-2.

Peripheral T cells are tolerant to self-antigens, which is achieved by limiting the expansion and activation of self-reactive T cells (1). In addition to the function of regulatory T cells (5), several intrinsic mechanisms have been discovered that either induce T cell anergy or limit T cell expansion (4), such as the GRAIL E3 ligase, which is induced in anergic T cells and inhibits IL-2 expression by targeting TCR signaling molecules (30). In addition, control of T cell growth and regulation of apoptosis are also important mechanisms to maintain self-tolerance (1). Egr-2 has been found to regulate expression of an E3 ligase (8), and now we show that Egr-2 directly activates p21cip1 expression in $CD44^{high}$ T cells and is involved in the control of Th1 and Th17 differentiation. Our data demonstrate that Egr-2 is an intrinsic regulator of effector T cells and is essential for the control of their proliferation and inflammatory activation in the absence of overt antigen stimulation. The fact that Egr-2 is expressed in effector, but not in naive T cells, and controls their proliferation in vivo without interfering with their responses to optimal TCR stimulation provides insight into how the threshold for T cell activation is regulated, thus enabling the activation and expansion of effector T cells only in response to optimal antigen stimulation and limiting the expansion of hypersensitive or potentially autoreactive cells. The development of systemic autoimmune disease in Egr-2 cKO mice with characteristics of human lupus makes this a good model for further studies into the mechanisms of lupus development and could potentially yield novel therapeutic strategies.

MATERIALS AND METHODS

Generation of conditional Egr-2 knock-out mice. Egr-2 cKO mice were generated by crossing floxed Egr-2 mice (obtained from P. Charnay, Institut National de la Santé et de la Recherche Médicale, Paris, France) (14) with hCD2-Cre transgenic mice in which Cre-induced recombination is detected only in T and B cells (13). The primer pairs (p1, 5′-agttgacagcccgagtccagtgg-3′; p2, 5′-gggagcgaagctactcggatacgg-3′; sense 5′-CCAACAACTACCTGTTCTGCCG-3′; antisense 5′-TCATCCTTGGCACCATAGATCAGG-3′) were used for genotyping of Egr-2-Loxp and iCre loci, respectively. The Egr-2 floxed mice are used as a control. $Rag2^{-/-}$ mice have been described before (13). All mice used in this study are on the C57BL/6 background or have been backcrossed at least three times to C57BL/6. All mice were maintained in the Biological Services Unit, Barts and The London School of Medicine, and used according to established institutional guidelines under the authority of a UK Home Office project license (Guidance on the Operation of Animals, Scientific Procedures Act 1986). UK home office approved our animal project license for this experiment.

Antibodies. FITC-conjugated antibodies to CD4 and CD62L; PE-conjugated antibodies to CD4, CD8, NK1.1, F4/80, CD25, and CD69; allophycocyanin (APC)-conjugated antibodies to CD44 and Annexin V; peridinin chlorophyll protein (PerCP)–conjugated antibody to CD3, PE-labeled anti-p-stat5, rat anti–mouse B220 antibody for immunohistochemistry, and antibody to CD3 (clone 145-2C11) and CD28 (clone 37.51) for stimulation were obtained from BD Biosciences. FITC-conjugated antibody to IFN-γ, PE-conjugated antibodies to IL-4, IL-2, and Foxp3 were bought from eBioscience. Rabbit anti–human CD3 antibody for immunohistochemistry was purchased from DAKO. FITC-conjugated donkey anti–rat IgG and Alexa Fluor 594–conjugated donkey anti–rabbit IgG second antibodies were obtained from Jackson Immunoresearch Laboratories. Egr-2 antibody was purchased from Covance.

Cell isolation and stimulation. Primary $CD4^+$ T cells were purified by positive selection using a MACS system (Miltenyi Biotec), with purity consistently >90%. The $CD4^+CD44^{high}$ and $CD4^+CD44^{low}$ T cells were sorted by FACS after staining with FITC-conjugated CD4, PE-conjugated CD44, and APC-conjugated CD25 antibodies (eBioscience). Cells were gated on $CD25^-$ cells to exclude activated cells and regulatory T cells. T cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, and 100 U/ml penicillin and 100 μg/ml streptomycin. Purified $CD4^+$ T cells were stimulated with 1 μg/ml CD3 and 1 μg/ml CD28 antibody-coated beads, uncoated beads, or IL-2 as indicated.

Proliferation. To measure proliferation, purified $CD4^+$ T cells (5×10^4 cells/200 μl) in 96-well plates were stimulated in triplicate. A total of 1 μCi of [^{3}H]TdR was added for the last 8 h of culture, and cells were harvested and subjected to scintillation counting to measure [^{3}H]TdR incorporation. To analyze the in vivo proliferation of T cells mice were fed with 0.8 mg/ml BrdU in drinking water for 9 d. The splenocytes were stained with PE-conjugated anti-CD4 or -CD8 antibody and APC-conjugated CD44 antibody, followed by staining with FITC-conjugated anti-BrdU antibody with the BrdU flow kit (BD Biosciences). The percentage of T cells that had incorporated BrdU was analyzed by FACS. The proliferation of adoptively transferred $CD4^+$ or $CD8^+$ T cells in WT C57BL/6 mice was analyzed by CFSE labeling. Purified $CD4^+$ or $CD8^+$ T cells from Egr-2 cKO or WT mice were labeled with CFSE and transferred into syngeneic 12-wk-old C57BL/6 mice. 3–4 wk later, $CD4^+$ or $CD8^+$ T cells were purified from the spleens of recipients and stained with PE-anti-CD44 antibody before analysis of CFSE staining.

Intracellular cytokine analysis. Splenocytes or $CD4^+$ T cells harvested from mice of different ages were stimulated with 20 ng/ml PMA plus 0.5 μg/ml ionomycin in the presence of Brefeldin A for 5 h. Cells were then stained for cell surface markers (CD4 and CD44), and intracellular cytokine staining was performed with the Fixation and Permeabilization kit and IFN-γ, IL-2, IL-4, and IL-17 antibodies in accordance with the manufacturer's instructions (eBioscience).

Quantitative real-time PCR. Real-time PCR was performed as previously described (31). In brief, total RNA was extracted from $CD4^+$ T cells before and after stimulation, or from $CD4^+CD44^{low}$ and $CD4^+CD44^{high}$ cells, using TRIzol (Invitrogen), and was reverse transcribed using oligo(dT) primers (GE Healthcare). Quantitative real-time PCR was performed on a Rotor-Gene system (Corbett Robotics) using SYBR green PCR master mix (QIAGEN). The primers used were as follows: Egr-2 sense 5′-CTTCAGCCGAAGTGACCACC-3′ and antisense 5′-GCTCTTCCGTTCCTTCTGCC-3′; p21 sense 5′-TTGCACTCTGGTGTCTGAGC-3′ and antisense 5′-GAGGACCAATCTGGGCTTGG-3′; β-actin sense 5′-AATCGTGCGTGACATCAAAG-3′ and antisense 5′-ATGCCACAGGATTCCATACC-3′; IL-2 sense 5′-GCATGTTCTGGATTTGACTC-3′ and antisense 5′-CAGTTGCTGACTCATCATCG-3′; IL-4 sense 5′-CAAACGTCCTCACAGCAACG-3′ and antisense 5′-CTTGGACTCATTCATGGTGC-3′; IL-17A sense 5′-AGCGTGTCCAAACACTGAGG-3′ and antisense 5′-CTATCAGGGTCTTCATTGCG-3′; IL-17F sense 5′-AACCAGGGCATTTCTGTCCC-3′ and antisense 5′-TTTCTTGCTGAATGGCGACG-3′; and IFN-g sense 5′-CCATCAGCAACAACATAAGC-3′ and antisense 5′-AGCTCATTGAATGCTTGGCG-3′.

The data were analyzed using the Rotor-Gene software. All samples were run in duplicate, and relative mRNA expression levels were obtained by normalizing against the level of β-actin from the same sample under the same program using the following equation: relative expression = $2^{(CT\beta\text{-actin}-CTtarget)} \times 10{,}000$.

Immunoblotting. Total cellular protein or cytosolic and nuclear extracts were prepared from $CD4^+$ T cells before and after stimulation, as previously described (9). Nuclear proteins were finally dissolved in 20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease and phosphatase inhibitors (Roche), and the aliquots were frozen at −80°C. Protein concentration was determined using the BCA method (Sigma-Aldrich) according to the manufacturer's instructions. Immunoblotting was performed as in our previous study (9).

EMSA. EMSA was performed as previously described (31). In brief, the p21 probe (5′-GGGCTGCCTCTGAGGGGGCGGGGC-3′) was labeled with [α-^{32}P]dCTP using Ready-to-Go DNA labeling beads (GE Healthcare) according to the manufacturer's instructions. This was then purified using ProbeQuant G-50 microcolumns (GE Healthcare), and binding reactions were performed with nuclear extracts from stimulated and unstimulated MF2.2D9 cells in 10 mM Hepes, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 μg poly(dI-dC), 10% glycerol, 0.5 mM $ZnCl_2$, and 5 mM spermidine. The reactions were incubated at room temperature for 40 min with double-stranded ^{32}P-labeled oligonucleotide. For supershift reactions, anti-Egr-2 and anti-Egr-3 antibody were added after 10 min of incubation. The samples were electrophoresed on 5% polyacrylamide gels in 0.5× TBE. The gels were dried under vacuum and exposed to autoradiographic film at –80°C.

Retroviral transduction. pBabe Egr-2, provided by K. Jessen (University College London, London, England) was transfected into the Phoenix packaging cell line using FuGene according to the manufacturer's instructions. Retrovirus containing supernatant was collected 48 h later. This was added to a RetroNectin (Takara)-coated plate and incubated at 32°C for 4 h. The plates were washed with PBS, and then MF2.2D9 murine T hybridoma cells, provided by K.L. Rock (University of Massachusetts Medical School, Worcester, MA) were added. Cells were expanded and GFP-positive cells were isolated by FACS.

ChIP. ChIP assays were performed according to the protocol supplied by Millipore. 10^8 Egr-2 and GFP-transduced MF2.2D9 cells were cross-linked with 1% formaldehyde for 10 min at room temperature. After quenching of formaldehyde with 125 mM glycine, chromatin was sheared by sonication. The size of sonicated chromatin was ∼300–1,000 bp as analyzed on agarose gels. Fragmented chromatin (500 μg) was precleared for 1 h with salmon sperm DNA/BSA-blocked protein A beads (GE Healthcare) and subjected to immunoprecipitation with specific anti-Egr-2 Ab (Covance) or control antibody at 4°C overnight. Immunocomplexes were recovered by incubation with blocked protein A beads and washed in low salt, and then high salt, and finally LiCl buffer. DNA was purified by phenol chloroform extraction and used as template for PCR with specific oligonucleotides p21 sense (5′-ATCGGTGAAGGAGTGGGTTGGTCC-3′), p21 antisense (5′-ACACCTCTCGGCTGCTGCAGTTGG-3′), SOCS3 sense (5′-TGTGTACTCAAGCTGGTGCAC-3′), and SOCS3 antisense (5′-CATACTGATCCAGGAACTCC-3′).

Histological and serological analysis. Tissues were fixed with 10% formalin in PBS and embedded in paraffin. Sections were stained with hematoxylin and eosin by standard methods. Histological examination of kidneys was done in a blind manner. Proteinuria was determined using reagent strips for urinalysis, URS-5K (Access Diagnostics Tests). For immunohistochemistry, kidney sections were stained with rabbit anti–human CD3 and rat anti–mouse B220. After washing, sections were further stained with Alexa Fluor 594–conjugated donkey anti–rabbit IgG and FITC-conjugated donkey anti–rat IgG secondary antibodies, followed by counterstaining with DAPI. For analysis of Ig deposits, 6 μm frozen kidney sections were processed as previously described (32) and stained with a Texas red–conjugated donkey anti–mouse IgG (Jackson ImmunoResearch Laboratory). Total serum IgG and Ig isotypes were captured with goat anti–mouse Ig, and concentrations were determined by ELISA with HRP-conjugated antibodies against IgG, IgG1, IgG2a, IgG2b, and IgM (Sigma-Aldrich). Reactivity of these antibodies was normalized for equivalent OD against total IgG and the corresponding isotype controls (Sigma-Aldrich).

The levels of anti-histone and anti-dsDNA antibodies in serum were measured according to published methods (33). In brief, 5 μg/ml Calf

thymus DNA or histones (Sigma-Aldrich) in PBS were coated at 0.2 ml/well in Immulon II microtiter plates (Dynatech Laboratories., Inc.). After overnight incubation at 4°C, wells were postcoated with 0.4 ml gelatin (1 mg/ml in PBS) for 2 h at 37°C. After washing, 0.2 ml of serum samples diluted 1:1,000–1:4,000 in 0.1% Tween, 1 mg/ml gelatin, and 0.5% BSA in PBS were added and incubated for 1.5 h at room temperature. After washing, HRP-conjugated rabbit anti–mouse Ig (Dako) diluted 1:4,000 in 0.1% Tween in PBS was added. After 1.5 h of incubation at room temperature, the wells were washed and substrate solution was added. The OD was then read with an automated spectrophotometer at 492 nm.

Adoptive transfer. T cell–depleted splenocytes were prepared from Egr-2 cKO and WT mice aged 8 mo using a Pan T cell isolation kit (Miltenyi Biotec), according to the manufacturer's protocol and $CD4^+$ T cells were isolated by positive selection with CD4 (L3T4) microbeads (Miltenyi Biotec). 10^6 $CD4^+$ T cells from Egr-2 cKO mice were mixed with 1.5×10^6 T cell–depleted splenocytes from WT mice or 10^6 $CD4^+$ T cells from WT mice were added to 1.5×10^6 T cell–depleted splenocytes from Egr-2 cKO mice. These mixtures were suspended in 100 µl of physiological saline and injected i.v. into the dorsal tail vein of female $Rag2^{-/-}$ mice at 12 wk of age. 75 d after transfer, serum antibodies against dsDNA and histones were measured.

Statistics. The Mann-Whitney *U* test was used. The normality of data distribution and the homogeneity of variances of the data were verified by the K-S Lilliefors and Cochran C tests, respectively.

Online supplemental material. Fig. S1 shows the results of genotyping of Egr-2 cKO mice and Egr-2 expression in B and T cells. Fig. S2 shows development of thymocytes in Egr-2 cKO mice comparing to WT mice. Fig. S3 shows the subgroups of lymphocytes in spleens of WT and Egr-2 mice. Fig. S4 shows ERK and STAT5 activation in T cells after TCR ligation. Fig. S5 shows the results of apoptosis analysis of CD4 T cells. Fig. S6 displays percentage of NK, macrophage, and B cells in spleens of WT and Egr-2 cKO mice. The online supplemental material is available at http://www.jem.org. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20080187/DC1.

We thank Dr. Patrick Charnay for providing Egr-2-loxp mice and Dr. Kristjan Jessen for pBabe Egr-2. We thank Mr. Steven Pash and Mrs. Christine Newton, Biological Science, Brunel University, UK for technical support. We also thank Dr. Matthias Merkenschlager Imperial College, London for advice on the Chip assay, and Dr. Gary Warnes for assistance with FACS analysis.

This work was supported by the Biotechnology and Biological Sciences Research Council and the Medical Research Council. D.C. Wraith is supported by Wellcome Trust.

The authors have no conflicting financial interests.

Submitted: 28 January 2008
Accepted: 7 August 2008

REFERENCES

1. Schwartz, R.H. 2003. T cell anergy. *Annu. Rev. Immunol.* 21:305–334.
2. Surh, C.D., O. Boyman, J.F. Purton, and J. Sprent. 2006. Homeostasis of memory T cells. *Immunol. Rev.* 211:154–163.
3. Appleman, L.J., and V.A. Boussiotis. 2003. T cell anergy and costimulation. *Immunol. Rev.* 192:161–180.
4. Lin, A.E., and T.M. Mak. 2007. The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells. *Curr. Opin. Immunol.* 19:665–673.
5. Germain, R.N. 2008. Special regulatory T-cell review: a rose by any other name: from suppressor T cells to Tregs, approbation to unbridled enthusiasm. *Immunology.* 123:20–27.
6. Anderson, G., P.J. Lane, and E.J. Jenkinson. 2007. Generating intrathymic microenvironments to establish T-cell tolerance. *Nat. Rev. Immunol.* 7:954–963.
7. Harris, J.E., K.D. Bishop, N.E. Phillips, J.P. Mordes, D.L. Greiner, A.A. Rossini, and M.P. Czech. 2004. Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells. *J. Immunol.* 173:7331–7338.
8. Safford, M., S. Collins, M.A. Lutz, A. Allen, C.T. Huang, J. Kowalski, A. Blackford, M.R. Horton, C. Drake, R.H. Schwartz, and J.D. Powell. 2005. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat. Immunol.* 6:472–480.
9. Anderson, P.O., B.A. Manzo, A. Sundstedt, S. Minaee, A. Symonds, S. Khalid, M.E. Rodriguez-Cabezas, K. Nicolson, S. Li, D.C. Wraith, and P. Wang. 2006. Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur. J. Immunol.* 36:1374–1385.
10. O'Donovan, K.J., W.G. Tourtellotte, J. Millbrandt, and J.M. Baraban. 1999. The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci.* 22:167–173.
11. Topilko, P., S. Schneider-Maunoury, G. Levi, A. Baron-Van Evercooren, A.B. Chennoufi, T. Seitanidou, C. Babinet, and P. Charnay. 1994. Krox-20 controls myelination in the peripheral nervous system. *Nature.* 371:796–799.
12. Carter, J.H., J.M. Lefebvre, D.L. Wiest, and W.G. Tourtellotte. 2007. Redundant role for early growth response transcriptional regulators in thymocyte differentiation and survival. *J. Immunol.* 178:6796–6805.
13. De Boer, J., A. William, G. Skavdis, N. Harker, M. Coles, M. Tolaini, T. Norton, K. Williams, K. Roderick, A.J. Potocnik, and D. Kioussis. 2003. Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *Eur. J. Immunol.* 33:314–325.
14. Taillebourg, E., S. Buart, and P. Charnay. 2002. Conditional, floxed allele of the Krox20 gene. *Genesis.* 32:112–113.
15. Mittelstadt, P.R., and J.D. Ashwell. 1999. Role of Egr-2 in up-regulation of Fas ligand in normal T Cells and aberrant double-negative lpr and gld T cells. *J. Biol. Chem.* 274:3222–3227.
16. Rengarajan, J., P.R. Mittelstadt, H.W. Mages, A.J. Gerth, R.A. Kroczek, J.D. Ashwell, and L.H. Glimcher. 2000. Sequential involvement of NFAT and Egr transcription factors in FasL regulation. *Immunity.* 12:293–300.
17. Balomenos, D., J. Martin-Caballero, M.I. Garcia, I. Prieto, J.M. Flores, M. Serrano, and C. Martinez-A. 2000. The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development. *Nat. Med.* 6:171–176.
18. Santiago-Raber, M.L., B.R. Lawson, W. Dummer, M. Barnhouse, S. Koundouris, C.B. Wilson, D.H. Kono, and A.N. Theofilopoulos. 2001. Role of cyclin kinase inhibitor p21 in systemic autoimmunity. *J. Immunol.* 167:4067–4074.
19. Arias, C.F., A. Ballesteros-Tato, M.I. Garcia, J. Martin-Caballero, J.M. Flores, C. Martinez-A, and D. Balomenos. 2007. p21CIP1/WAF1 controls proliferation of activated/memory T cells and affects homeostasis and memory T cell responses. *J. Immunol.* 178:2296–2306.
20. Swirnoff, A.H., and J. Milbrandt. 1995. DNA-binding specificity of NGFI-A and related zinc finger transcription factors. *Mol. Cell. Biol.* 15:2275–2287.
21. Curtsinger, J.M., D.C. Lins, and M.F. Mescher. 1998. CD8+ memory T cells (CD44high, Ly-6C+) are more sensitive than naive cells to (CD44low, Ly-6C-) to TCR/CD8 signaling in response to antigen. *J. Immunol.* 160:3236–3243.
22. Theofilopoulos, A.N., and F.J. Dixon. 1985. Murine models of systemic lupus erythematosus. *Adv. Immunol.* 37:269–390.
23. Andrews, B.S., R.A. Eisenberg, A.N. Theofilopoulos, S. Izui, C.B. Wilson, P.J. McConahey, E.D. Murphy, J.B. Roths, and F.J. Dixon. 1978. Spontaneous murine lupus-like syndromes: clinical and immunopathological manifestations in several strains. *J. Exp. Med.* 148:1198–1215.
24. Lawson, B.R., R. Baccala, J. Song, M. Croft, D.H. Ko, and A.N. Theofilopoulos. 2004. Deficiency of the cyclin kinase inhibitor p21(WAF-1/CIP-1) promotes apoptosis of activated/memory T cells and inhibits spontaneous systemic autoimmunity. *J. Exp. Med.* 199:547–557.
25. Luger, D., P.B. Silver, J. Tang, D. Cua, Z. Chen, Y. Iwakura, E.P. Bowman, N.M. Sgambellone, C.C. Chan, and R.R. Caspi. 2008. Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category. *J. Exp. Med.* 205:799–810.

26. Porter, C.M., and N.A. Clipstone. 2002. Sustained NFAT signaling promotes a Th1-like pattern of gene expression in primary murine CD4+ T cells. *J. Immunol.* 168:4936–4945.
27. Liu, X.K., X. Lin, and S.L. Gaffen. 2004. Crucial role for nuclear factor of activated T cells in T cell receptor-mediated regulation of human interleukin-17. *J. Biol. Chem.* 279:52762–52771.
28. Sela, U., M. Dayan, R. Hershkoviz, O. Lider, and E. Mozes. 2008. A peptide that ameliorates lupus up-regulates the diminished expression of early growth response factors 2 and 3. *J. Immunol.* 180: 1584–1591.
29. Kang, H.K., M. Liu, and S.K. Datta. 2007. Low-dose peptide tolerance therapy of lupus generates plasmacytoid dendritic cells that cause expansion of autoantigen-specific regulatory T cells and contraction of inflammatory Th17 cells. *J. Immunol.* 178:7849–7858.
30. Anandasabapathy, N., G.S. Ford, D. Bloom, C. Holness, V. Paragas, C. Seroogy, H. Skrenta, M. Hollenhorst, C.G. Fathman, and L. Soares. 2003. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity.* 18:535–547.
31. Anderson, P., A. Sundstedt, E. Massey, S. Li, D. Wraith, and P. Wang. 2005. IL-2 overcomes the unresponsiveness, but fails to reverse the regulatory function of antigen-induced Treg cells. *J. Immunol.* 174:310–319.
32. Li, S., E.E. Kaaya, H. Feichtinger, G. Biberfeld, and P. Biberfeld. 1993. Immunohistochemical distribution of leucocyte antigens in lymphoid tissues of cynomolgus monkeys (Macaca fascicularis). *J. Med. Primatol.* 22:357–362.
33. Kotzin, B.L., and E. Palmer. 1987. The contribution of NZW genes to lupus-like disease in (NZB x NZW)F1 mice. *J. Exp. Med.* 165:1237–1251.